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# Hematopoeitic System, Recombinant Products, and Clinical Applications

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## Hemoglobinopathies

In 1956, by identifying sickle hemoglobin through peptide mapping, Ingram made fundamental observations that formed the basis for a true molecular approach to human disease.[1] Subsequently, the sickle cell hemoglobin abnormality was traced to an abnormal peptide and eventually to a single amino acid change in the β chain of adult hemoglobin. Later it became apparent that this single amino acid substitution had resulted from a single base change in the coding DNA.[2] In the decade that followed, increasing medical and scientific attention was focused on a form of anemia known as thalassemia, which was first described in 1925 by Cooley, a pediatrician.[3] Quite early, its association with patients of Mediterranean heritage was apparent. When techniques for the in vitro study of globin and globin chain synthesis became available, it was evident that an inherited defect in hemoglobin synthesis formed the basis for this condition. As systems for cell-free RNA translation and hybridization became available, it was found that α-thalassemia was associated with deficiency in α-globin production related to actual deletion of α-globin structural genes. β-Thalassemia, associated with deficiency of β-globin, was apparently almost never caused by a deletion but was due to other structural β-globin changes. The various thalassemias, particularly β-thalassemia, are a major public health problem throughout the world. The application of new molecular approaches had made their etiology easier to study as knowledge about these disorders has increased.

### Human Globin Genes

Human globins are coded for by one or two structural genes.[4] All normal human hemoglobin molecules are composed as tetramers of two α-like and two β-like globin polypeptide chains. Genes for α-like chains are grouped together on the short arm of chromosome 16, whereas the β-like genes are situated together on

the short arm of chromosome 11. The β gene complex from the 5′ to the 3′ direction includes the single embryonic ε gene; two fetal (γ) genes, Gγ and Aγ; a pseudogene 4β1; the δ gene; and the adult β gene.[5] The order of genes within the DNA reflects embryonic–fetal–adult expression. During very early development, the ε-globin gene is expressed, followed by the γ-globin genes during fetal life. At 32 weeks of gestation, expression of the β gene is augmented and that of the γ genes is down-regulated except for a very low level of γ-gene expression that extends even into adult life.[6] The exact mechanisms that control these changes in hemoglobin gene expression are still not clearly understood. The complete β-globin gene complex extends over 60 kb of DNA and has been isolated and examined by cloning techniques.[5] Two intervening sequences, or introns, separate the coding regions of the β-globin gene into three exons.

## β-Thalassemias

The general term β-thalassemia indicates a rather diverse group of conditions in which there is a deficiency of β-globin production relative to α-globin within erythrocytes. Subjects who are heterozygous for β-thalassemia—namely those in whom only one β-globin allele is abnormal—are clinically normal. Patients carrying two β-thalassemia genes are designated as homozygous and generally are severely anemic, requiring lifelong medical supervision. A spectrum of severity can be recognized in which patients severely handicapped by the disorder may have $\beta^0$-thalassemia, in which no β-globin is detected, and $\beta^+$-thalassemia, in which β chains are detectable but in markedly reduced amounts.

The thalassemia syndromes can be considered in terms of the specific mutations involved. At least 30 different mutations have been identified on the basis of molecular cloning, DNA sequence analysis, and studies of mutant gene segments. Most of these mutations are relatively simple and involve single base substitutions or small deletions or insertions either directly within or just upstream of the β-gene sequences. Mutations influence major aspects of gene function, including transcription, RNA processing, and RNA translation to final gene products. Several sorts of mutations that result in β-thalassemia are diagrammed in Figure 8.1. It can be seen that these mutations involve transcription, nonsense, frameshift, deletions, insertions, and substitutions involving splicing regions of the gene.

In contrast to simple thalassemias, the complex β-thalassemias involve abnormalities in expression of other β-like genes, either with a thalassemic component or with persistent expression of fetal hemoglobin into adult life. Complex β-thalassemias can be due to rather extensive DNA deletions. For instance, in typical persistence of fetal hemoglobin into adult life, (termed HPFH) (as recognized in blacks) deletion of both β- and δ-globin genes is found. At the same time both γ-globin genes are expressed at a high level. In δβ-thalassemia the β gene is completely deleted and most, but not all, of the δ gene is absent. Several examples of globin gene deletions with designations of comparable clinical syndromes are shown in Figure 8.2. The precise mechanisms leading to these various deletions are not yet known. Rarely, in some clinical syndromes associated with an increase in fetal hemoglobin production, all genes in the β complex are inactive or deleted in a clinical picture termed γδβ-thalassemia. The entire β-globin gene complex may be deleted from the chromosome,[7] or the μ, γ, and δ genes and the 5′ region of the β gene may be deleted.[8,9] In such instances there is some evidence for altered methylation of DNA in the region upstream from the β-globin sequences.[10]

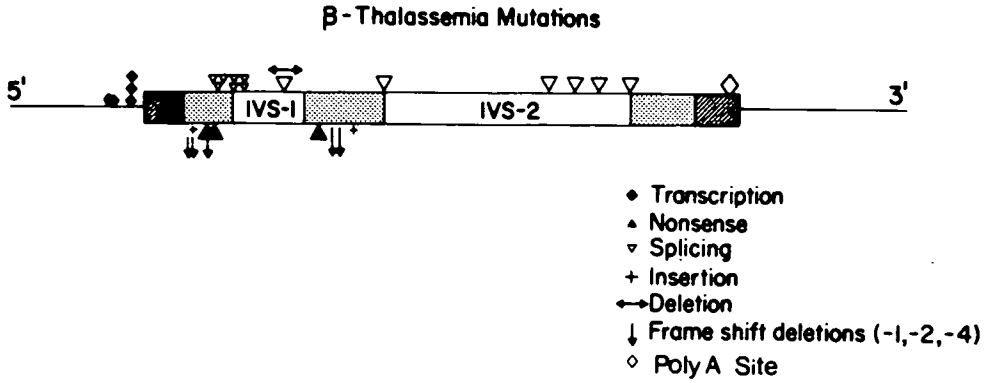
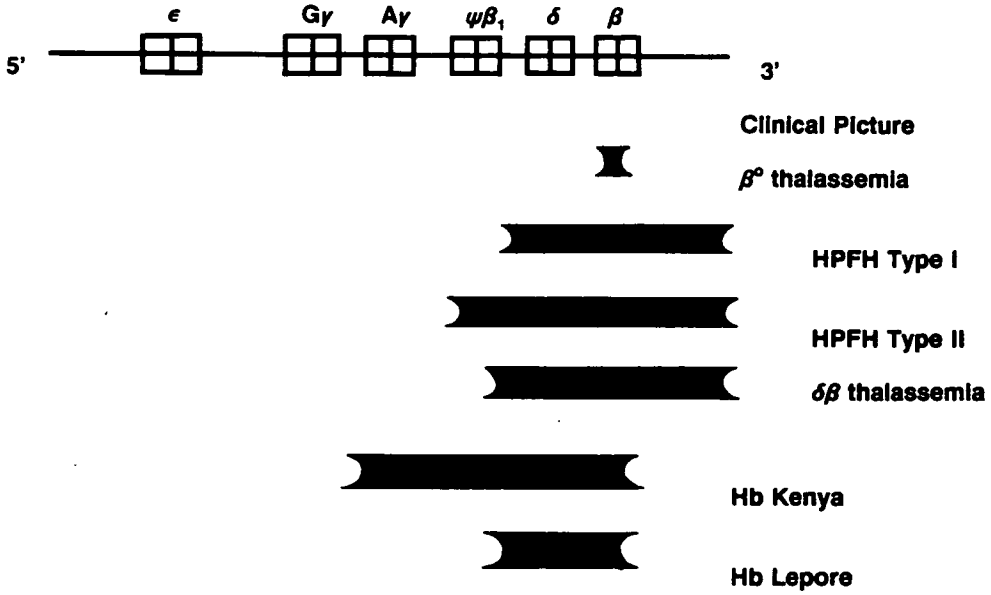



Figure 8.1 Location of β-thalassemia mutations in the β-globin gene. Specific mutations are indicated along the linear map of the β-gene with a symbol corresponding to its effect on β-gene expression. Key: ◆, transcription mutant; ↓, frameshift deletion; ↔, 25-bp deletion; ◇, 3′ RNA cleavage mutant; ▲, nonsense mutant; +, frameshift insertion; ▽, RNA splicing mutant. *Reproduced from Orkin and Kazazian*[14] *with permission.*

As noted above, most cases of β-thalassemia result from deletions or point mutations of β-globin genes as shown in Figure 8.1. In some patients the mutations can be identified directly using conventional β-region probes that recognize such deletions or mutations by changes in restriction enzyme recognition sites.[11–13] Direct analyses of chorionic villous tissue for fetal DNA in the first trimester permits an early diagnosis. Because more than 30 different mutations are now known to be associated with β-thalassemia,[14] in order to identify a mutation it is necessary to synthesize a pair of oligonucleotide probes, one of which is completely complementary in sequence to the normal gene and the other complementary to its abnormal counterpart. Such an approach has been applied to several Mediterra-

Figure 8.2 Deletions in the β-globin gene cluster. The respective regions of deletion for the clinical designations listed at the right are shown as dark bars. *Adapted from Orkin and Kazazian,*[14] *with permission.*



nean populations at risk.[15] In this analysis the two oligonucleotide probes synthesized were for the G → A mutation at position 110 within the first intervening sequence and for the C → T mutation at codon 39 of the β-globin gene. Each oligonucleotide consisted of 19 bases, half of which were completely homologous to the normal β-globin gene sequence and the other half to the thalassemia gene sequence. DNAs prepared from peripheral blood samples obtained from a large number of families of Mediterranean origin known to contain thalassemia cases were cut by restriction enzymes and hybridized under stringent conditions to $^{32}$P-labeled oligonucleotide probes. Using these two probes for the most common thalassemic mutations in the populations being studied, it was found that analysis of 75% of the parents by this technique could predict the likelihood of the children being homozygous for the β-thalassemia defects. In such instances, the success of this technique in prenatal diagnosis varies with the region or country in which it is applied. A single oligonucleotide probe for the $\beta^0$39 mutation would detect 95% of all thalassemia mutations in Sardinia, where the genetic heterogeneity for this gene presumably was more restricted.[16] Extension of this basic screening technique has indicated that specific β-thalassemia mutations are correlated with certain well-defined β-globin haplotypes.[17] Thus, to be complete, a screening process should probably include probes for all possible mutations, which would of necessity include probes for all rare β-thalassemia mutations. These exploratory studies provide important guidelines for prenatal diagnostic screening for thalassemias using fetal DNA analyses. In most populations at risk, there are usually one or two particularly common mutations together with a number of rarer forms of β-thalassemia. At present, unless automation systems become more widely available, covering the latter possibility with a large enough battery of oligonucleotide probes may prove too expensive and time consuming for widespread implementation. Even if the multiple oligonucleotide probe approach eventually becomes feasible, it probably would have to be combined with fetal blood sampling in order to detect the relatively small number of cases in which one or both parents are carriers for rare β-thalassemia mutations.

## Amplified Single-Copy DNA

A great technical step forward was made in studies of human genomic mutations with the introduction and application of the polymerase chain reaction (PCR), which allows tremendous amplification of specific DNA regions of interest.[18–20] This method utilizes the *Taq* DNA polymerase enzyme and results in marked expansion of amplified gene products, which can then be sequenced and analyzed in comparative studies (Figure 8.3). The PCR method, which has now been applied to numerous human genetic diseases, is capable of amplifying selected DNA sequences 220,000-fold by 20 cycles of DNA polymerase I reactivity.[18] Unusual β-thalassemia mutations have been studied by this method using direct nucleotide sequencing of amplified single-copy DNAs.[21] These analyses uncovered two previously unrecognized mutations and clearly defined additional mutations present among other patients studied. Because of its speed and the exact information it yields, direct genomic sequencing can in some instances bypass the need for determination of haplotypes in the prenatal diagnosis of disorders such as β-thalassemia.

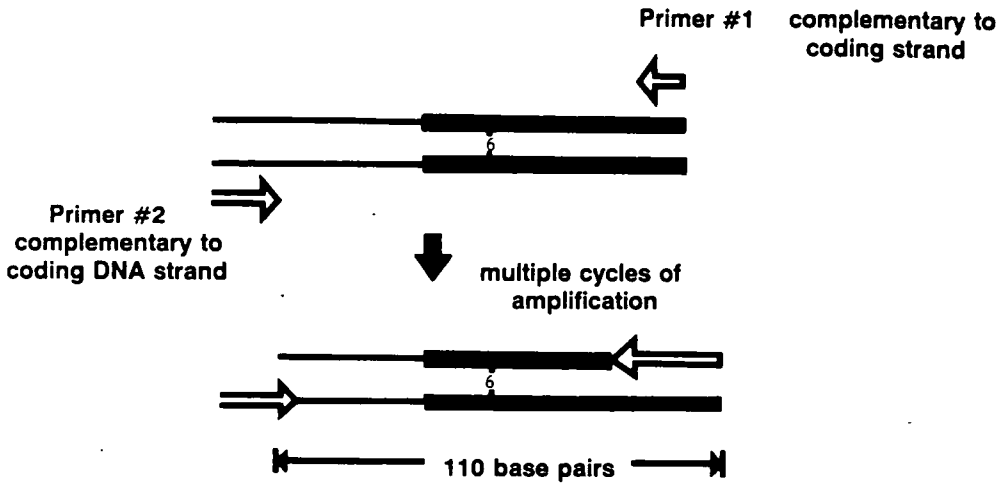


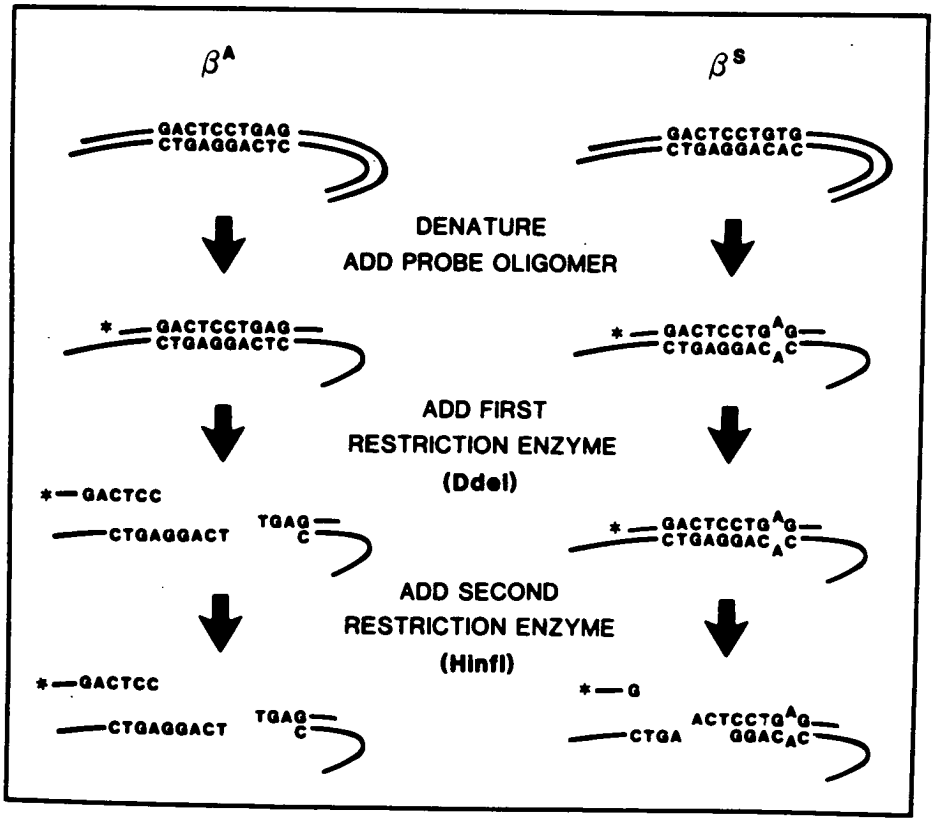

Figure 8.3 (Top) The two strands of the β-globin gene (dark lines) and its relation to polymerase chain reaction primers 1 and 2, which flank the sixth codon. The arrows show the direction in which the primers are extended by the polymerase reactions. Primers are provided as 20-base oligonucleotide DNA segments complementary to sequences that directly flank the target codon. Each oligonucleotide primer was extended using the Klenow fragment of *Escherichia coli* DNA polymerase I, so that each extension product serves as the template for a subsequent round of replication primed by the other primer. (Bottom) The major amplification products after many cycles of amplification. These products are discrete DNA segments (110 base pairs) that represent the genomic DNA between the outside or 5′ end of the primers and the sequences including the β codon of interest.

## Sickle Cell Anemia

The method of DNA polymerase I amplification of β-globin DNA sequences has been particularly useful in the case of sickle cell anemia. This disease, which is associated with homozygous sickle cell gene expression, is relatively common in the United States, since it is estimated that 3000 pregnancies per year involve risk of producing a homozygous *ss* child. Several methods have been introduced for prenatal testing for sickle cell anemia, including recombinant DNA analysis.[22,23] Direct identification of DNA point mutations permits greater specificity and clinical certainty than the use of indirect methods involving linkage or haplotype analysis, which often require studies of parents and other family members.[14,24] The method of in vitro enzymatic amplification of genomic β-globin sequences was used for diagnosis of sickle cell anemia.[18] Previous methods that required DNA from fetal trophoblastic tissue were technically challenging procedures that usually took several days and could be done in only a few major centers. Application of the DNA polymerase I PCR amplification technique to the diagnosis of sickle cell anemia again involved hybridization of the amplified target DNA sequences with short synthetic oligonucleotide primers labeled at the 5′ end. Hybridized DNA was then digested sequentially with two different restriction enzymes,[25] which produced digestion products of different lengths, depending on whether the $\beta^A$ or $\beta^S$ allele was present in the digested target DNA. The restriction DNA fragments were separated by electrophoresis and then identified by autoradiography.[26] Embury et al.[26] used a new method of DNA analysis to differentiate the normal GAG codon at $\beta^6$ from the GTG sickle mutation. The method involved amplification of target DNA $\beta^6$ nucleotide sequences with a 40-nucleotide hybridization probe complementary

to the $\beta^A$ noncoding strand, following by labeling at the 5′ end with $^{32}P$. The annealed hybrids were digested with *Dde*I and then *Hinf*I restriction enzymes. The selectivity of the method was based on the fact that the single base mismatch in the enzyme target restriction site of the hybridization probe–target DNA segment inhibited cleavage by the restriction endonuclease. Thus, sequential digestions by the two restriction enzymes produced DNA fragments of a size distribution that was characteristic of either $\beta^A$ or $\beta^S$ sequences. This differential process is shown in Figure 8.4. Using such an enzymatic amplification step followed by the restriction fragment length analysis, it was possible to identify accurately DNAs from subjects with AA, AS, SS, sickle β-thalassemia, and heterozygous β-thalassemia. Examples of this sort of analysis are shown in Figure 8.5. The major advantages of this method are that it is rapid and relatively sensitive. The high degree of amplification obtained with the PCR method (now automated) allows genetic analysis of very small

**Figure 8.4** Steps in oligomer restriction. The top two diagrams show DNA molecules containing the $\beta^A$ and $\beta^S$ alleles, respectively. The two diagrams next to the top show the noncoding strands of these molecules after denaturation and annealing to the $\beta^A$-specific probe labeled at the 5′ end (*). The $\beta^A$ target strand is perfectly complementary to the 40-base probe, but the $\beta^S$ target strand contains a single-base mismatch, shown as a bubble (designated by the unaligned A). The two diagrams that are third from the top demonstrate the results of digesting the two duplexes with *Dde*I. The duplex with $\beta^A$ target sequence contains a *Dde*I site, and its cleavage generates a 5′-labeled 8-base product that dissociates from the duplex. The duplex with $\beta^S$ target sequences is not cleaved because of the mismatch within its *Dde*I site. Diagrams at the bottom demonstrate that subsequent digestion with *Hinf*I did not affect the 8-base cleavage product of $\beta^A$ but generated a labeled 3-base product from the duplexes that contain $\beta^S$ target sequences. *Reproduced from Embury et al.*[26] *with permission.*



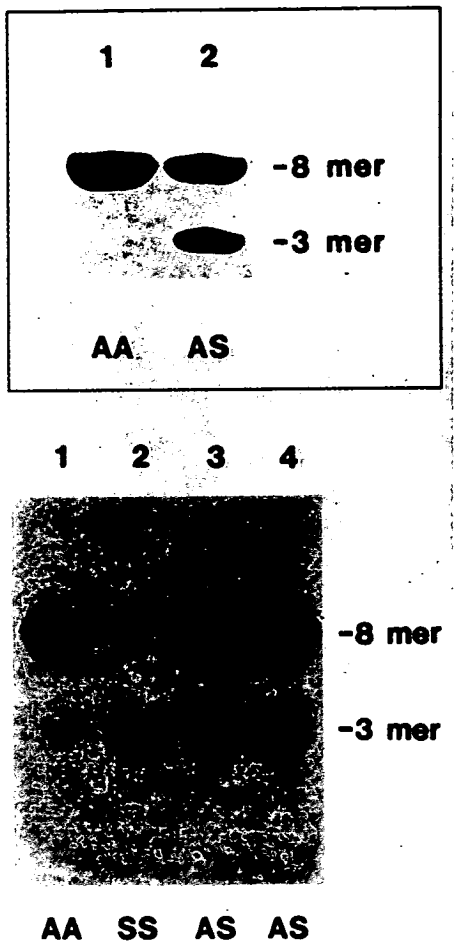



Figure 8.5 Autoradiograms showing results of polymerase chain reaction oligonucleotide restriction for diagnosis in pregnancy at risk for hemoglobin SS. (A) Results for couple 1. DNA from the cultured amniocytes of the fetus yielded only the 8-base cleavage product diagnostic of hemoglobin AA (lane 1). DNA from the leukocytes of the mother yielded both 8-base and 3-base products (lane 2), confirming the diagnosis of hemoglobin AS. The uncleaved 40-base probe is not shown in this or subsequent autoradiograms. (B) Results for couple 2 and for analysis of control DNA samples (hemoglobin AA and hemoglobin SS). Hemoglobin AA DNA produced to only an 8-base cleavage product (lane 1), and hemoglobin SS DNA only a 3-base product (lane 2). DNA from the leukocytes of the mother and amniocytes of the fetus resulted in both an 8-base and a 3-base product (lanes 3 and 4, respectively), demonstrating the hemoglobin AS genotype in each. *Reproduced from Embury et al,*[26] *with permission.*

amounts of fetal material, eliminating the previous need for cell culture. Moreover, detection of the hemoglobin SC genotype (which is indistinguishable from the AS genotype by direct restriction analysis of the $\beta^6$ codon affected by the single point sickle mutation) can also be done using the amplification $\gamma$ double restriction digest procedure.

Another approach that can be used to detect the $\beta^C$ allele is to prepare sequence-specific oligonucleotide probes to analyze amplified genomic DNA.[19,27] The general PCR DNA amplification procedure is a promising approach that will be applicable to a number of other genetic diseases. The only requirements are that the mutation being sought affects a known restriction enzyme site and that the adjacent nucleotide sequences are also known.

## α-Thalassemia

In the normal state there are four copies of the α-globin gene on human chromosome 16. The most common cause of α-thalassemia is gene deletion; the α-thalassemia syndromes have been best defined in Asian populations, in which four clinical states of increasing severity have been described. These are shown in Table 8.1. A nonthalassemic individual has four α-globin structural genes per diploid cell; in α-thalassemia 1 and hemoglobin H disease, two and one α-globin genes, respectively, are still intact, and in homozygous α-thalassemia associated with hy-

Table 8.1 The α-Thalassemia (α-Thal) Syndromes (Asian Populations)

| Syndrome | Genotype | Clinical manifestation | Number of α-globin genes[a] |
|---|---|---|---|
| Silent carrier state | α-Thal-2 | None | 3 |
| Heterozygous α-thalassemia | α-Thal-1 | Microcytosis | 2 |
| Hemoglobin-H disease | α-Thal-1 × α-Thal-2 | Microcytosis, hemolysis | 1 |
| Homozygous α-thalassemia | α-Thal-1 × α-Thal-1 | Hydrops fetalis (fatal) | 0 |

[a] Number of globin genes in α-thalessemia-2 is inferred. All others have been shown by hybridization.

drops fetalis at birth, no α-globin genes remain intact.[28,29] Initially, homozygous α-thalassemia was detected using DNA hybridization from cultured amniotic fluid fibroblasts.[30] Subsequently this approach was improved to differentiate between homozygous α-thalassemia and hemoglobin H disease or α-thalassemia trait by using $^{35}$S-labeled probes and slot blot analysis to measure actual α-globin gene dosage directly.[31] This assay is particularly useful because it does not require Southern blotting, restriction enzyme digestion, and gel electrophoresis. Moreover,the slot blot technique uses $^{35}$S-labeled probes, which have a much longer half-life than those labeled with $^{32}$P. In Southeast Asian countries such as Thailand, where approximately 15,000 hydropic pregnancies occur each year, application of this method should make prenatal diagnosis of α-thalassemia much more feasible than in the past.

## Hemoglobin E

In 1954 Itano and co-workers[32] described a hemoglobin with previously unrecognized electrophoretic behavior in a child of Hindu and Mediterranean parentage. About the same time, Chernoff et al.[33] reported similar findings in patients studied in Thailand. This new abnormal hemoglobin was called hemoglobin E. It was found to result from the substitution of glutamic acid by lysine at position 26 in the β chain. At present, hemoglobin E may be the cause of the most prevalent hemoglobinopathy in the world, affecting an estimated 20 million individuals.[34] Because of the influx of Southeast Asian refugees into the United States and Canada since the Vietnam conflict, hemoglobin E is being recognized with increasing frequency.[35]

The single point mutation at codon position 26 of the β-globin gene involves substitution of an adenine for a guanine (GAG → AAG); the amino acid substitution occurs at the sensitive $\alpha_1\beta_1$ contact site, a region involved with other unstable hemoglobins.[36] Hemoglobin E is unusual because a single DNA base substitution produces both an unstable hemoglobin and erythrocytes with an apparent $\beta^+$-thalassemia phenotype. In homozygous individuals, hemoglobin E is associated with normal or mildly decreased hemoglobin levels in association with microcytosis; however, in doubly heterozygous individuals, the combination of hemoglobin E with β-thalassemia can produce an illness as severe as that seen with thalassemia major. The $\beta^E$ mRNA appears to be unstable during erythrocyte maturation and abnormal qualitative and quantitative processing of $\beta^E$ mRNA occurs, including

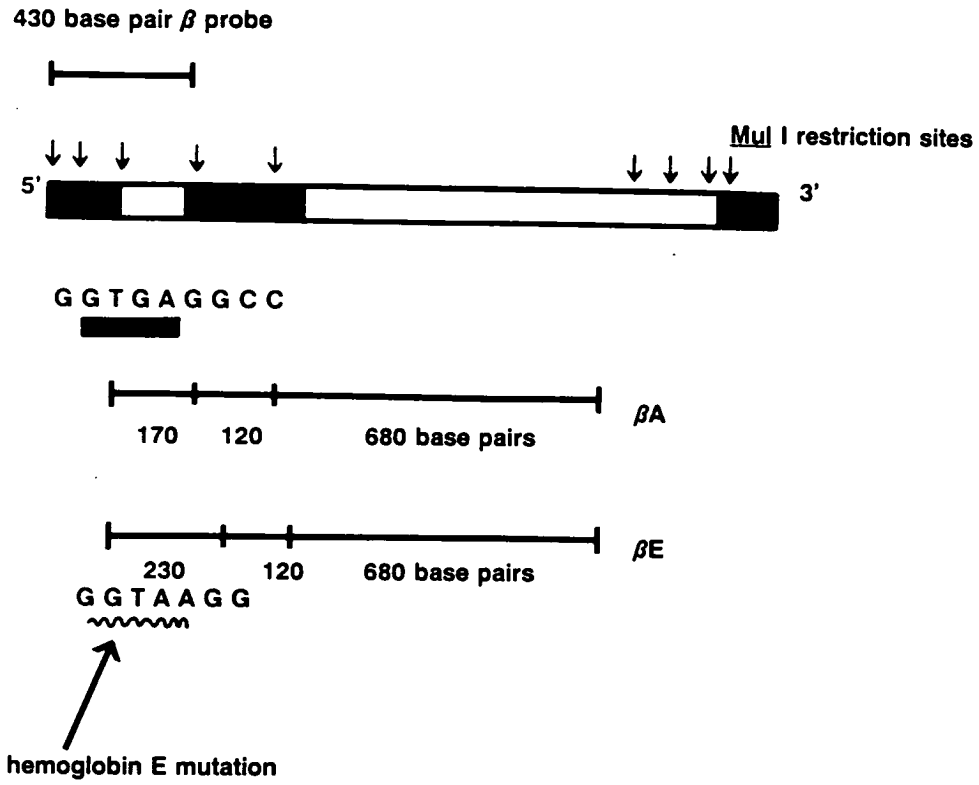



Figure 8.6 Schematic map of the β-globin gene showing the restriction cleavage sites (arrows) for *MnlI*. After digestion and hybridization with the 430-bp β probe shown above, because the $\beta^E$ chain shows the AAGG mutation at the *MnlI* restriction site, a 230-bp fragment is produced instead of the 170-bp fragment seen in the normal $\beta^A$-globin digest.

abnormalities in mRNA splicing and production of an unstable $\beta^E$ mRNA product.[37] Homozygous hemoglobin E is a clinically benign condition that does not appear to be associated with a survival disadvantage. Oxidant medications should be used with some caution because there have been a few reports of hemolytic anemia after use of drugs such as dapsone or para-aminosalicylic acid.

Direct detection of hemoglobin E using restriction endonuclease mapping has been reported[38] after digestion with the restriction enzyme *MnlI*. This method takes advantage of the fact that the hemoglobin E single point mutation destroys the enzyme cleavage site at nucleotide sequences 3'-GGAG-5'. Figure 8.6 presents a schematic map of the β-globin gene along with the recognition sites for *MnlI* restriction endonuclease. When normal $\beta^A\beta^A$ DNA is digested with *MnlI* and hybridized with the 430-bp probe, fragments of 60, 170, 120, and 680 bp are observed. With the mutation of hemoglobin E, the cleavage site for *MnlI* is removed and the 170-pb fragment increases in size to 230 bp. This method provides a convenient way to identify the abnormality.

## Prospects for the Future

Because of the severe consequences of β-thalassemia and sickle cell anemia, considerable investigation and effort have gone into ways to treat the disease directly. Approaches that have been considered include reactivation of γ-globin gene expres-

sion and introduction of normal β genes into marrow of patients with severe homozygous forms of β-thalassemia or sickle cell anemia.

An approach that has long seemed reasonable is to influence the fetal switch process whereby during normal development γ gene expression is modulated down and β gene expression is increased late in gestation. Since relative hypomethylation of the 5′-flanking region of the γ genes in fetal erythroid cells has been noted,[39,40] an attempt was made in baboons to test the effects of 5-azacytidine, a compound known to produce extensive demethylation.[41] Treatment with this drug produced a considerable increment in hemoglobin F levels in the baboons, suggesting that drug treatments such as this might be used to modulate the fetal switch experimentally.[42] Small numbers of β-thalassemia and sickle cell anemia patients were treated with 5-azacytidine and dramatic increments in HbF expression were noted.[43–45] At the same time that HbF expression increased, hypomethylation of the γ-globin gene 5′-flanking regions was recorded. A strange feature of treatment with this drug was that the response appeared to be so rapid; after about 48 hours reticulocytes with the capacity to manufacture HbF were noted. It was therefore thought that the recorded modulation could have reflected cytotoxic effects of the drug involving preferential killing of cycling erythroid cells, permitting emergence of other precursors more committed to HbF synthesis.

Other cytotoxic drugs such as cytosine arabinoside and hydroxyurea, which do not specifically inhibit DNA methylation, have also been used experimentally, first in primates[46] and then in a few patients with sickle cell anemia.[47] After more extended use of 5-azacytidine in larger numbers of patients, enthusiasm for its protracted use was chilled by reports that the drug was probably a carcinogen in laboratory animals.[48] Hydroxyurea was employed because one of the theories of 5-azacytidine action suggested that it led to recruitment of early erythroid precursors capable of increased HbF production during recovery of early marrow suppression. Moreover, hydroxyurea was easily administered and reported to be noncarcinogenic in limited studies.[49] Since its initial trials, considerable long-term experience with this drug has been obtained, particularly in patients with sickle cell anemia.[50] In one study positive responses in several patients treated for longer than 2 years were encouraging and associated with signs of clinical improvement. It was noted that the HbF response was seen within 2 to 3 weeks but establishment of a large proportion of circulating F cells required treatment for a month or longer. Examples of positive responses are shown in Figure 8.7. During serial studies, several patients showed an increased number of chromosomal structural rearrangements or deletions, causing concern that patients placed on such regimes should avoid conception and pregnancy. One of the practical factors determining response to hydroxyurea was the patient's baseline level of reticulocyte production, which is an inherited trait and ranges from 4% to 50%. Patients with the highest initial baseline levels of recitulocyte production achieved the highest mean F reticulocyte counts during treatment.[50] The risk of carcinogenesis from long-term hydroxyurea treatment in humans is unknown. Studies to date have unproved safety but probably involve a relatively small risk.

## Gene Therapy—Thalassemia Models

With the advent of recombinant methods and gene cloning, reintroduction of genes into various cell types and animals by transfection techniques has now become possible. One of the first successful attempts resulted in specific expression of a

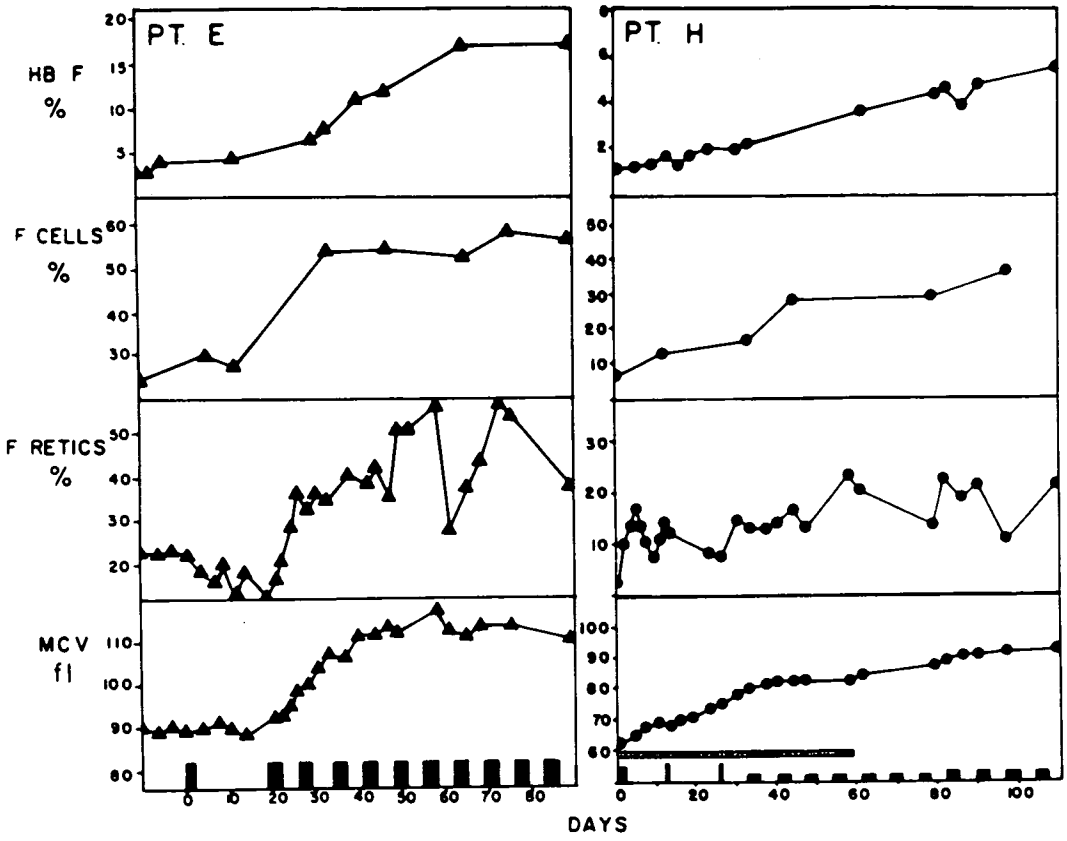


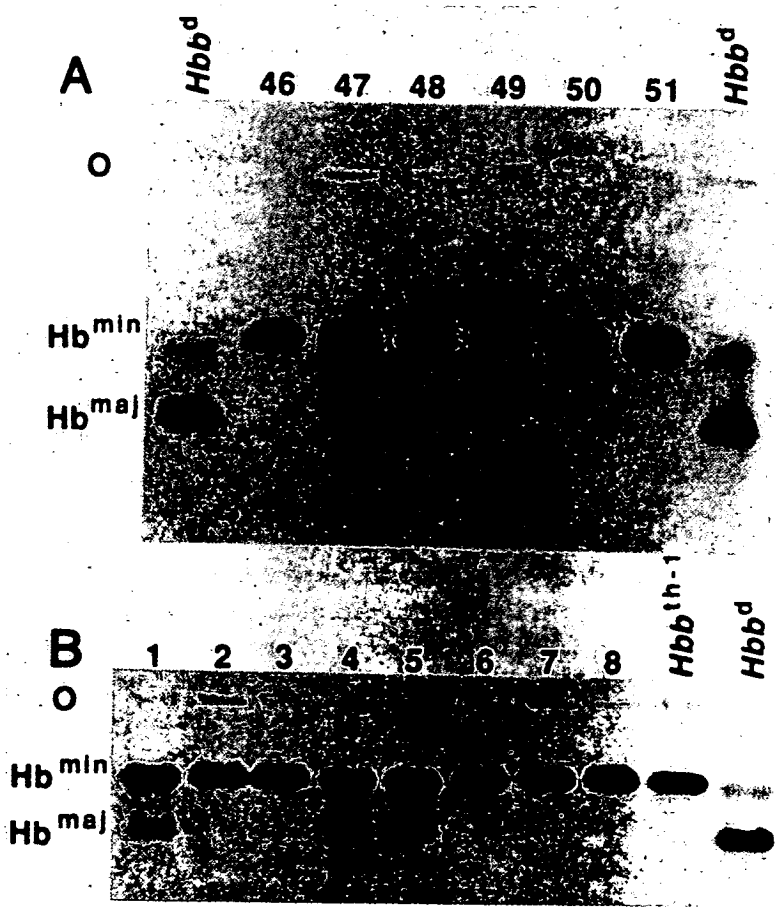

Figure 8.7 Response of patients E and H to hydroxyurea. Patient E had been transfused, and H was iron deficient before therapy. Black bars represent HU and the stippled bar $FeSO_4$ treatment. *Reproduced from Charache et al,[50] with permission.*

foreign β-globin gene in erythroid cells of transgenic mice.[51] Transgenic mice carrying a hybrid mouse–human adult β-globin gene showed gene expression predominantly within erythroid tissues. Most remarkable in these early studies was the fact that regulatory sequences closely linked to the transfected β-globin genes were able to specify correct patterns of tissue-specific expression in the experimental mice. More recently it was reported that a mouse model of β-thalassemia was corrected by transfer of cloned β-globin genes into the mouse germ line. The cloned mouse $\beta^{maj}$-globin gene or the cloned human β-globin gene was introduced into mice that were deficient in β-globin synthesis because of deletion of the $\beta^{maj}$-globin gene. Both artificially introduced genes produced functional β-globin chains leading to relative correction of the anemia and associated red blood cell abnormalities.[52] These interesting experiments were made possible by the availability of a murine model of thalassemia, which arose spontaneously in a mouse of the β-globin haplotype *Hbb*$^d$, which includes the two adult β-globin genes $\beta^{maj}$ and $\beta^{min}$. In the mutation, *Hbb*$^{th-1}$, the $\beta^{maj}$ gene is entirely deleted but the $\beta^{min}$ gene remains intact. Animals homozygous for this deletion produced a reduced amount of β-globin (as in human thalassemia) and consequently also had a hemolytic anemia similar to that in human β-thalassemia of moderate severity. Although correction of genetic deficiencies by transfer of genes into the germ line itself instead of directly into developed somatic cells would not be a feasible strategy for human gene therapy, it was attempted as a trial in this mouse model. Two approaches were used,

first with the cloned normal mouse $\beta^{maj}$-globin gene and second with the cloned normal human β-globin gene. In the first experiment, a 7.0-kb *Eco*RI fragment containing the mouse $\beta^{maj}$-globin gene was microinjected in the presence of calcium ions into homozygous $Hbb^{th-1}$ mouse zygotes and the eggs were transferred to foster mothers. Of 129 transferred eggs, 10 mice were born. Of these, two mice carried multiple copies of the original microinjected gene as determined by Southern blot analysis. Moreover, when peripheral blood samples from the transgenic mice were examined, one of the two mice was found to be producing a significant amount of the $\beta^{maj}$-type hemoglobin. These results are shown in Figure 8.8. When the transgenic male mouse was subsequently mated to homozygous $Hbb^{th-1}$ females, several of the progeny were found to produce $\beta^{maj}$ hemoglobin, indicating that they had inherited and were capable of expressing the original microinjected gene (Figure 8.8). Although the ratio of $\beta^{maj}$ to $\beta^{min}$ hemoglobin is 4:1 in normal homozygous $Hbb^{b}$ mice and 1:1 in heterozygotes, in the transgenic mice the ratio was found to be 1:2. Therefore, the transfected genes were producing fewer globin chains than a natural endogenous $\beta^{maj}$-globin gene.

Similar successful gene transfer experiments were performed in which a 7.7-kb

**Figure 8.8** Expression of the cloned $\beta^{maj}$-globin gene in transgenic mice. (A) Cellulose acetate electrophoresis of cystamine-modified hemolysates from two normal mice ($Hbb^{d}$) and six mice (MB46–MB51) developed from homozygous $Hbb^{th-1}$ zygotes that were microinjected with $\beta^{maj}$ gene. Mice MB47 and MB51 are transgenic. (B) A similar analysis of eight offspring (1–8) of mouse MB47 mated to a homozygous $Hbb^{th-1}$ female mouse. *Reproduced from Costantini et al,[52] with permission.*



human β-globin gene fragment was transfected into the thalassemic mice. These experiments demonstrate the feasibility of transfection into germ-line DNA. Moreover, the thalassemia was essentially eliminated in the transgenic line carrying the human β-globin gene and partially corrected in the transgenic line carrying the cloned $\beta^{maj}$-globin gene. These differences were attributed to relative differences in level of expression of the two gene products. The actual levels of expression have been observed to vary considerably, apparently as a function of the chromosomal locus at which specific genes are integrated.[51-53] Cloned β-globin genes transferred into the erythroid cells of a thalassemic patient by any route would likely be expressed at variable levels from cell to cell. This potential problem will probably not be completely overcome until there is a much improved general understanding of regulatory mechanisms involving globin genes.

The idea that transgenic transfer therapy may be successful in human disorders of hemoglobin production was given impetus by the report by Dzierzak et al.[54] of successful expression of human β-globin genes in murine bone marrow transplant recipients reconstituted with retrovirus-transduced stem cells. A number of previous studies used retroviral vectors to introduce exogenous DNA sequences into target cells such as hematopoietic stem cells. In many of the systems previously studied there was no convincing evidence for significant levels of expression of the introduced genes. Until recently, all of the retroviral vectors employed with bone marrow cells were designed to promote eventual expression of inserted complementary DNA sequences. In most cases the vectors contained either viral transcriptional signals in the long terminal repeat (LTR) of the provirus or specific nonretroviral transcriptional control sequences to produce active chimeric RNAs. One reason for previous difficulties could be that the transcriptional sequences used lack elements specifically needed for hematopoietic stem cells to ensure gene expression at adequate levels during stem cell development. Another possibility is that the chimeric mRNAs generated by retroviral vectors produced structures that were abnormally processed or degraded prematurely.

In the studies by Dzierzak et al.,[54] three recombinant genomes were used. These are shown in detail in Figure 8.9. In two instances a 3-kb *Hpa*I-*Pst*I segment of human DNA encoding the human β-globin gene was inserted into the *Bam*HI site of the vectors in such a way that the direction of the β-globin transcription was *opposite* that of the proviral transcription. The third recombinant genome was constructed by insertion of a 4.1-kb *Hpa*I-*Xba*I DNA segment of the human β-globin gene into pSV(Z)neo. An additional 1.1 kg of 3'-flanking nucleotide sequence containing an enhancer element was thought to be responsible for increased levels of human β-globin expression in transgenic mice. Bone marrow cells from C3H/HeJ female mice were prepared and infected in vitro. Then $5 \times 10^4$ to $4 \times 10^6$ cells were injected intravenously into lethally irradiated male mice, and 1 to 2 months after transplantation peripheral blood samples were obtained from each recipient. DNA from blood samples was analyzed for the presence of proviral sequences using Southern blots. These studies confirmed successful engraftment in a substantial proportion of animals, indicating that the engrafted recipients expressed mRNA as well as human β-globin product in their erythroid cells. Tissue specificity of expression was also examined in the engrafted mice. It was found that human β-globin RNA expression was restricted to cell populations of erythroid lineage. These exciting preliminary results were encouraging about the prospect for transduced somatic cell engraftment as potential therapy for human disorders such as the hemoglobinopathies. The cDNA sequences of chromosomal human β-globin were

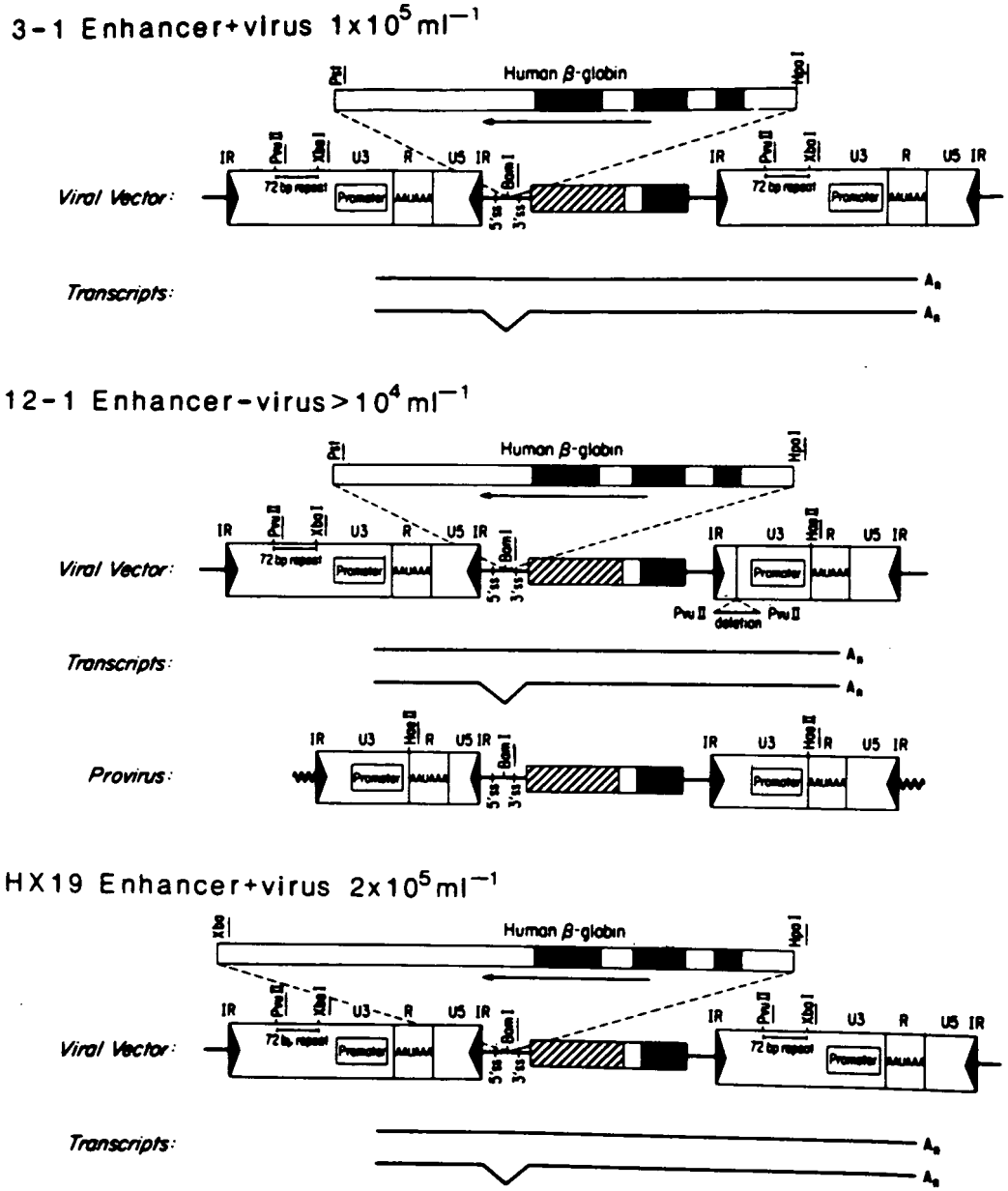



Figure 8.9 Structure of the human β-globin gene recombinant retroviruses and detection of recombinant proviral genomes in bone marrow–engrafted mice. HX19 was constructed by introducing a 4.1-kb *HpaI-XbaI* fragment of human DNA containing the human β-globin gene into pSV(X)neo. Analysis of the three cell lines used in this study for helper virus in the XC assay yielded no plaques after multiple passages. *Reproduced from Dzierzak et al.*[54] *with permission.*

encoded as an intact gene that was expressed exclusively in erythroid cells of all transplant recipients, whether or not the viral transcriptional control signals in the vector were still present. Although expression of the transferred human β-globin gene was extremely tissue specific, the absolute level of β-globin transcription was relatively low. This could be improved by using additional sequences 50 kb upstream and 20 kb downstream of the β-globin structural gene, which dramatically

boost expression in transgenic mice containing a single copy of the sequences.[55] Clearly, more work is now necessary to understand how the retroviral sequences directly influence transcription of β-globin sequences. It may be possible that *cis*-acting sequences of nucleotides within β-globin gene segments may actually activate proviral transcription. The results of the experiments of Dzierzak et al.[54] have a number of important implications for studies of gene expression in hematopoietic cells and the potential of human gene transfer therapy. Much previous effort had been directed at hematopoietic stem cell infection and the use of cDNA expression vectors. However, the work described above relied on the use of transcriptional signals normally used in hematopoietic cell differentiation. Perhaps gene therapy in the disorders of hemoglobin may not be as difficult or impossible as was thought a few years ago.

## Sickle Cell Transgenic Models

An animal model of sickle cell disease has become available through the efforts of two different groups of investigators.[56,57] Greaves and co-workers[56] used the dominant control region sequences from the human β-globin locus together with human α- and $\beta^S$-globin genes in transgenic mice. The animals fell into two categories: one group that expressed the constructs at low levels because of deletions or various degrees of mosaicism and a second group that were fully transgenic and expressed the transgenes at high levels.[58,59] Among the fully transgenic mice, several showed high levels of human α-globin and $\beta^S$-globin mRNA in peripheral blood and showed a near-balanced synthesis of human α- and $\beta^S$-globin RNA. Analysis of peripheral blood hemolysates showed a large hemoglobin component that focused at the same isoelectric point as HbS from a sickle cell patient. These transgenic mice were not anemic, but in vitro sickling of their erythrocytes could be produced using the standard sodium metabisulfite assay. Some of the transgenic progeny showed sickling in vivo; others did not. One mouse with 83% $Hb^S$ had normal somatic development, no obvious manifestations of disease, and no evidence for hemolytic anemia. No clear explanation for these findings was apparent; however, the authors suggested that the residual 17% of normal mouse hemoglobin may have been enough to protect the animal from disease manifestations. The same situation sometimes arises in the human disease state, where heterozygotes for $\beta^S$ and HPFH also show HbS levels ranging from 65% to 90% but no anemia, normal red cell indices, and mostly normal reticulocyte counts.

In the second transgenic mouse model,[57] human α- and $\beta^S$-globin genes were inserted downstream of erythroid-specific, deoxyribonuclease I superhypersensitive sites coinjected into fertilized mouse eggs. Transgenic lines showed synthesis of human hemoglobin S. The same animals were then bred to β-thalassemic mice to reduce endogenous mouse globin levels. When red blood cells from these mice were subjected to deoxygenation, more than 90% showed the sickling phenomenon. Of great interest, these mice had decreased hematocrit values, elevated reticulocyte counts, and splenomegaly—all similar to human patients with sickle cell disease.

As more information is generated with these new models of sickle cell disorder it should be possible to test new drugs or therapeutic strategies with respect to affecting or ameliorating the underlying disease.

### Stimulation of Fetal Hemoglobin Using Recombinant Erythropoietin

As noted earlier, stimulation of fetal hemoglobin production may help patients with sickle cell anemia by inhibiting sickling. Fetal hemoglobin appears to be limited in adults to a subpopulation of erythrocytes termed F cells.[60–62] Erythroid progenitor cells may have the ability to form F cells, but most of them fail to do so when the kinetics of erythroid differentiation and maturation are normal. However, when progenitor cells are pushed into their terminal differentiation prematurely, as in sudden bone marrow expansion, increased numbers of F cells may be formed. Erythropoietin produced by recombinant techniques has been used to stimulate F cell production in baboons.[63] The recombinant erythropoietin was administered to anemic baboons with the expectation that high doses would produce rapid erythroid regeneration. The animals were kept anemic by daily phlebotomy. These studies indicated that doses of 1500 to 3000 IU of erythropoietin rapidly produced substantial increments in F reticulocytes in both anemic and nonanemic animals. Levels of F reticulocytes increased from 1 to 2% to 40 to 50% after such treatment. Such stimulation of F cell production might be beneficial in the treatment of patients with sickle cell disease. If erythropoietin stimulates F cells in sickle cell disease, treatment spaced at 1- or 2-week intervals could result in accumulation of F cells in high enough numbers to inhibit sickling in vivo. Currently, it is estimated that about 20% hemoglobin F per red blood cell is required to protect against sickling.[64] The baboons treated with recombinant erythropoietin had levels of 12% to 26% hemoglobin F per red blood cell. Long-term human trials of this mode of therapy are in progress.

## Coagulation Factors

### Hemophilia A and B

Classical hemophilia or hemophilia A is a sex-linked disorder that occurs in approximately 20 per 100,000 males. In the blood, factor VIII:C is associated with another protein called von Willebrand factor VIII–related antigen. However, factor VIII:C is principally responsible for the functional clotting activity of the intrinsic complex, generically termed factor VIII. Injection of factor VIII preparations obtained from normal donor blood is currently the principal mode of therapy for hemophilia patients. These factor VIII preparations may carry a certain degree of risk for non-A, non-B hepatitis, and human immunodeficiency virus (HIV), or AIDS virus, has been shown to contaminate various plasma concentrates or preparations containing factor VIII used in the treatment of hemophilia. Most cases of hemophilia A are due to novel mutations of the factor VIII gene.[65] The relatively high frequency of hemophilia A in comparison to other autosomal clotting disorders is the result of localization of this gene on the X chromosome, so that only one affected chromosome is sufficient to produce the disease in males. Several decades ago the mean age of death of hemophiliacs was estimated to be less than 20 years; however, between 1950 and 1970 research on and improved understanding of the disorder led to successful treatment, first with whole plasma and later with fresh plasma concentrates. Human plasma from normal volunteer donors is an inefficient and relatively labile source of this protein, whose concentration is usually 100 to 200 ng/ml. Currently used commercial factor VIII–enriched plasma concentrates have usually been produced from pooled plasmas representing material derived in

some instances from thousands of donors. Thus such products are associated with potential undesirable effects of transmitting hepatitis, particularly non-A, non-B, as well as HIV infection.

## Recombinant Factor VIII

For these reasons it seemed urgent to provide factor VIII derived directly by recombinant techniques. This was first successfully accomplished in 1984 using factor VIII clones recovered from a bacteriophage λ genomic library.[66–69] The cloning strategy started with a λ phage cDNA library prepared from an individual with four X chromosomes (karotype 49,XXXXY). Initially the cDNA library was screened with a 36-base oligonucleotide probe synthesized to represent appropriate complementary nucleotides based on a sequenced peptide derived from highly purified factor VIII. When the initial hybridization procedure was successful, the clones containing clearly overlapping segments were expanded to cover 28 kb of the human chromosome. Later the clones were further amplified to include 200 kb of the human genome and the entire factor VIII gene. The structure of the human factor VIII gene is shown in Figure 8.10. The DNA sequence of the complete mRNA coding portion of the factor VIII gene, which spanned 9 kb, was then determined. Remarkably, in the initial analyses only two nucleotide differences between the genomic and the cDNA sequences were found within the entire 8860 nucleotides compared. These data suggested a much lower level of sequence polymorphism than had been documented in other genes and were consistent with similar findings related to other X chromosome loci.[70] The complete structural and sequence definition of the human factor VIII gene was a remarkable technical and scientific achievement, since at the time, in 1984, it was the largest gene yet characterized, including nearly 186 kb of the entire human X chromosome.

After the cloning of the gene for factor VIII, cDNA clones containing the complete 2351-amino-acid sequence for human factor VIII were isolated and used to produce biologically active functional factor VIII in cultured mammalian cells. When the recombinant work on factor VIII was begun, the exact site of synthesis was not known, although the liver was considered the most likely primary source. Also, because the concentration of factor VIII was known to be extremely low (200 ng/ml), or about 1/2,000,000 the concentration of serum albumin), it was not likely that cDNA libraries produced from mRNA of a tissue such as the liver would yield any clones producing factor VIII. For this reason the strategy noted briefly above was adopted. First, a genomic clone that expressed a portion of the sequenced part of the factor VIII protein was obtained. From this an exon-containing restriction fragment could be used to identify suitable tissue or cellular sources of factor VIII mRNA by Northern blotting or other hybridization procedures. Then mRNA from the latter tissue sources could be used to make cDNA clones, which could in turn be screened with previously cloned genomic fragments. With this approach, comparative nucleotide sequence analysis could be used to obtain overlapping genomic clones that would eventually include the entire factor VIII gene. Even if a direct tissue source of mRNA could not be found, exons from the genomic clones could be identified and spliced together using simian virus 40 (SV40) recombinant exon expression plasmids. In fact, the successful cloning and expression of factor VIII utilized information obtained from all of these approaches.

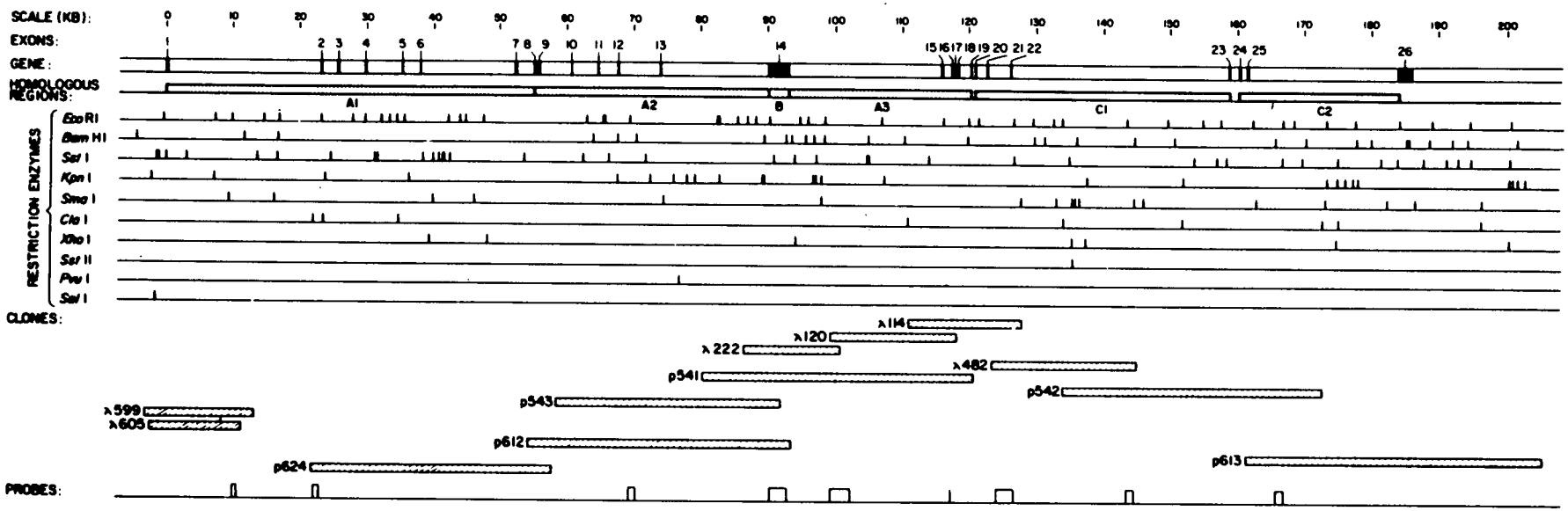



**Figure 8.10** Map of human factor VIII gene. The structure of the gene is schematically represented by an open bar; the 26 exons are filled-in areas drawn to scale. The direction of transcription is from left to right. The size scale in kilobases is drawn above the gene; the initiating ATG is positioned at nucleotide 1. Immediately below the gene are the extent of the triplicated A domain, the unique B domain, and the duplicated C domain. The locations of the recognition sites for the 10 restriction enzymes used to map the factor VIII gene are given in the next series of lines. Crosshatched boxes represent the extent of human genomic DNA contained in each λ phage and cosmid clone. Other clones that encompass the same regions are not shown. No genomic clones span the 8.5 kb of intron located between clones λ599 and p624. Other *Eco*RI, *Bam*HI, or *Sst*I sites may lie in this region. The extent of this gap was determined by probing genomic Southern blots with single copy fragments isolated from the 3′ end of λ599 and the 5′ end of p624. The bottom line shows the locations of synthetic oligonucleotide or restriction fragment probes used in the genomic cloning; from left to right, these are the two probes used to map the uncloned region, three 5′ walk probes, the original synthetic 36-mer probe 8.3, and three 3′ walk probes. *Reproduced from Gitschier et al,*[66] *with permission.*

### *cDNA Sequence*

When completely assembled and sequenced, the nucleotide arrangement of factor VIII predicted a polypeptide of 2351 amino acids that included a 19-amino-acid signal region, similar in some respects to the glycoprotein peptide hormones discussed in Chapter 7. Once the primary sequence was established, computer-aided search for internal homologies showed that the factor VIII sequence included three types of domains: a triplicated region of 330 to 370 amino acids referred to as the A domain, a unique B domain of 983 amino acids, and a duplicated C region of 150 amino acids. The arrangement of regions within the single long polypeptide chain was A1-A2-B-A3-C1-C2. Moreover, most of the potential asparagine-linked sites for glycosylation predicted by the primary sequence occurred within the B region. Of interest was the fact that the factor VIII protein sequence showed remarkable homology with the sequence of ceruloplasmin, a copper-containing protein that is known to behave as an acute phase reactant.

## Expression of the Factor VIII Gene

The factor VIII gene was assembled and recombinant tailoring was performed to prepare it for expression[69] by placing it between a tandem SV40 early promoter/adenovirus-2 major late promoter and the hepatitis B virus surface antigen gene polyadenylation sequences. In addition, the plasmid contained a murine dihydrofolate reductase cDNA transcribed by an SV40 early promoter to provide a detectable selected marker when expressed in mammalian cells. The final assembled plasmid is shown in Figure 8.11. This plasmid was transfected into a hamster kidney cell line using the calcium phosphate coprecipitation method. To determine whether the transfected factor VIII gene was expressed in the kidney cell line, RNA was extracted from the cells and analyzed by Northern blot hybridization. These experiments clearly showed positive results; moreover, radioimmune assays of the hamster kidney cell line supernatants were positive for factor VIII. These results provided evidence for successful expression of the factor VIII gene. Coagulation assays of the factor VIII product itself indicated that it was active because it improved the clotting activity of plasma from patients with hemophilia A from 120 to 65 seconds and the activity was neutralized by monoclonal antibodies to factor VIII. These studies represented a monumental recombinant DNA achievement in the successful cloning and production of a trace coagulation protein essential for normal body function. Although these initial definitive experiments were carried out a number of years ago, we are still anticipating full-scale production of recombinant factor VIII. The initial work with the genetically engineered material showed a very low level of expression. Technical improvements have now been implemented and full-scale production of recombinant factor VIII is awaited with much enthusiasm. Effective large-scale tissue culture and purification methods need to be developed to ensure production of a high-purity product.

## Genetic Mapping and Diagnosis of Hemophilia

Because hemophilia is the most common inherited bleeding disorder in humans, affecting one male in 10,000, it is important to be able to ascertain carriers of the disease and to assay fetal samples for diagnosis and genetic counseling. Initial studies directed at this problem built on the knowledge of the complete nucleotide

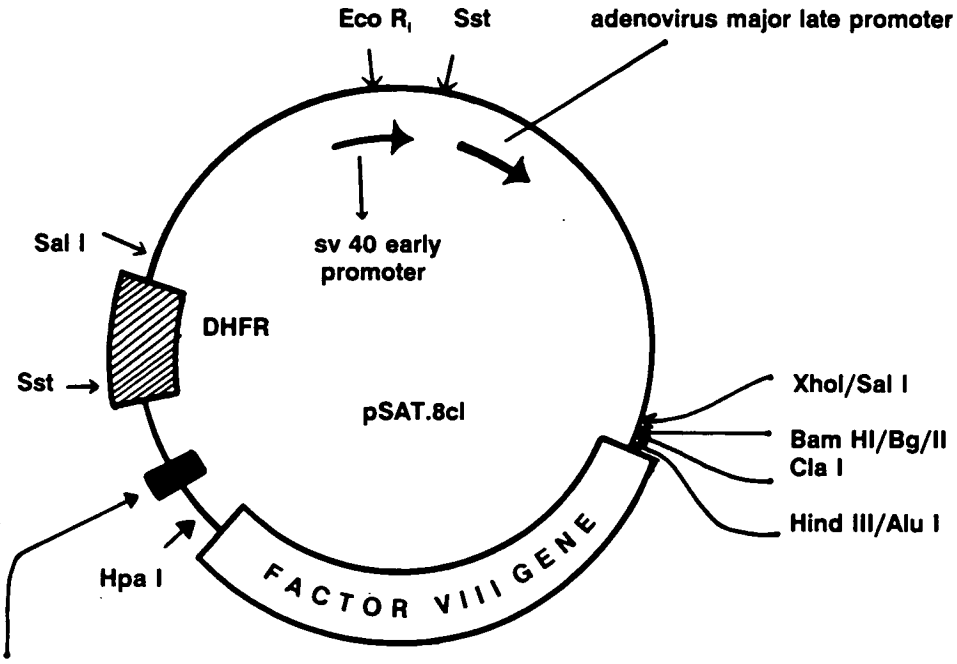



Figure 8.11 Simplified schematic diagram of full-length factor VIII expression plasmid derived from modification of pBR322. The factor VIII and dihydrofolate reductase genes are shown, as well as the tandem SV40 (early) and adenovirus major (late) promoters. The region between the *Sal*I and *Eco*RI restriction sites contains the ampicillin resistance gene. This plasmid was introduced or transfected into a hamster kidney cell line by the calcium phosphate coprecipitation method.

sequence and organization of the factor VIII gene. Early work showed an identifiable polymorphism revealed by DNA restriction digests using the enzyme *Bcl*I,[71] which could be used diagnostically in about 42% of families. Examples of this sort of analysis are shown in Figure 8.12. Interestingly, this study showed a relative paucity of restriction fragment length polymorphisms (RFLPs) with the factor VIII probe. As noted earlier, there are suggestions that DNA polymorphism may in general be more scarce in the human X chromosome than in autosomes. Within the entire 9000 base pairs making up the mRNA coding region of factor VIII genes sequenced from two individuals, only two nucleotide differences were found.[68,69]

Additional studies of individual hemophiliac patients began to turn up a pattern reminiscent of the β-thalassemia story, as it was shown that the disease stems from a heterogeneous collection of genetic lesions at different vulnerable sites within the nucleotide sequence of the gene for factor VIII. Moreover, because the disease can be fatal if untreated, new mutations must be constantly occurring to account for the observed frequency in the general population. Also, there appears to be a spectrum of disease severity, suggesting that a number of different individual mutations are involved in producing the disorder in individual patients.[71,72] As a corollary to this, only about 15% of hemophiliacs generate high levels of antibody to factor VIII. Studies of individual sequence mutations within patients' factor VIII genes have now confirmed that a number of different point mutations, including

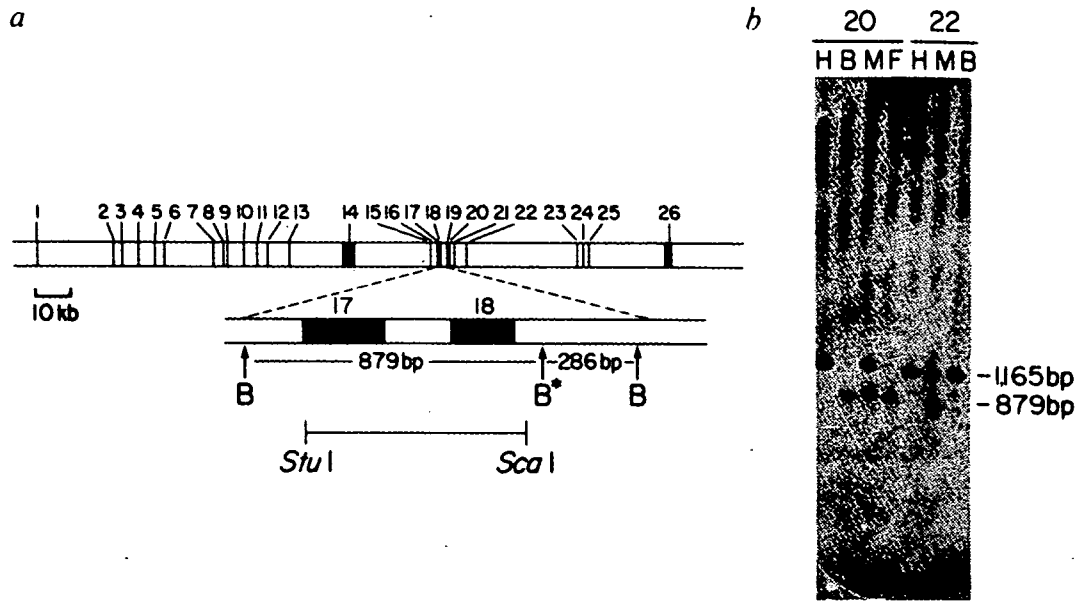



**Figure 8.12** Position of the *BclI* polymorphism within the factor VIII gene and an example of its usefulness in the diagnosis of hemophilia. (a) The factor VIII gene, containing 26 exons, is shown schematically. Below it is an expanded portion of the gene covering exons 17 and 18; the positions of the *BclI* sites (B) in the gene are indicated. The presence or absence of the middle site (in the intron just 3′ of exon 18 and marked by an asterisk) is responsible for the two alleles of 879 bp and 1165 bp. The location of the 647-bp *StuI/ScaI* genomic probe fragment is also shown. (b) A Southern blot of *BclI*-digested DNA (5 mg per lane) from members of two different families affected by hemophilia was hybridized to the 647-bp *StuI/ScaI* genomic probe. Family 20 consists of a hemophiliac son (H), whose brother (B) is not a hemophiliac, his heterozygous mother (M), a known obligate carrier of hemophilia, and father (F). Family 22 consists of a hemophiliac (H), his heterozygous mother (M), and his brother (B), who is also a hemophiliac. Blood samples from both patients and families were obtained from the Royal Free Hospital, London. *Reproduced from Gitschier et al,*[71] *with permission.*

in some instances partial deletions, are associated with the disease.[72] In these studies the restriction enzyme *TaqI* proved particularly useful for detecting differences in the DNA of individual hemophiliacs. The recognition site for *TaqI* contains the CpG dimer, which is the major methylation site for human DNA, and these CpG dimers are probably mutation "hot spots" secondary to subsequent deamination of methylcytosine to thymidine, resulting in C → T transitions.[73] Examination of the sequence and general structure of the factor VIII gene indicated that five of the seven *TaqI* sites in coding exons could be converted to nonsense mutations by such a C → T transition. Therefore, when 92 hemophiliac DNA samples were digested with *TaqI*, case-specific *TaqI* site mutations were discovered in four severe hemophiliacs.[72] Of great interest was the finding that each mutation site was different. In one instance loss of a specific *TaqI* site was found, and in another a *TaqI* site was introduced by mutation in an intron. Moreover, two cases of partial deletions of the factor VIII gene were detected in this study. Again, these deletions occurred in case-specific sites. Thus, unlike the single point mutation in sickle cell anemia, the genetic causes of hemophilia A appear to encompass a wide spectrum of mutations and deletions in vulnerable regions of the factor VIII gene.

## Sites for Factor VIII Synthesis

Cellular locations for factor VIII synthesis have been sought for some time. A number of studies suggested the liver as the primary site of synthesis, but other sources such as spleen and lung were also implicated.[74–78] Localization of factor VIII antigen by immunological techniques did not distinguish storage sites from actual sites of production. When the gene for factor VIII was cloned, hybridization probes for mRNA in tissues readily became available and were applied to a spectrum of human tissues.[78] Factor VIII mRNA was found in isolated human hepatocytes, in spleen, and in numerous other tissues including lymph nodes and kidney, but not in leukocytes or cultured endothelial cells. During this study spleens and hepatic tissues from several patients with severe hemophilia were studied for factor VIII mRNA. Surprisingly, normal levels were noted, suggesting that some cases of functionally severe hemophilia may be caused by other molecular defects affecting RNA or protein production and that only in some cases is the disease due to complete absence of factor VIII mRNA.

## Examining the Spectrum of Hemophilia A Mutations

As work with the cloned factor VIII gene has progressed, various groups have pinpointed the cause of hemophilia in an increasing number of cases. It was long suspected that the disorder resulted from a diverse spectrum of mutations in the large factor VIII gene, and this has been borne out. Examination of several hundred hemophilia A patients by DNA blot hybridization has documented at least seven distinct mutations as shown in Figure 8.13.

As methodology and more experience are gained with genetic screening techniques after the gene corresponding to a disease has been isolated, RFLPs present within or very close to the gene can be employed for genetic screening. A human DNA fragment (St.14) that recognizes a very polymorphic locus in the q28 region of the X chromosome containing the hemophilia locus has been identified.[79] This probe appeared to be a useful tool for early prenatal diagnosis using chorionic biopsy material. The probe had previously been mapped to the distal end of the X chromosome, a short genetic distance from the locus for hemophilia A. Analysis of 13 families showed striking linkage of St.14 and hemophilia in 12, indicating high coefficients of linkage. Use of this probe was estimated to give an accuracy of 93%. Moreover, use of the probe combined with biological assays could provide an accuracy of at least 96% in carrier analysis. Although statistically useful, this approach is still not a foolproof method for carrier or prenatal identification, and additional refinements are clearly needed.

The polymerase amplification technique has also added a new dimension of accuracy and technical improvement. This technique has been utilized by Kogan et al.[80] with intragenic factor VIII polymorphisms recognized by *XbaI* and *BclI* and the *Taq* polymerase. It was found that an additional dividend could be achieved by raising the temperature of the polymerase reaction from 37°C to 63°C, which produced a vast majority of fragments of the correct size and sequence. This occurred because at the higher temperature priming occurred only when template and primer were exactly matched, whereas at 37°C the oligonucleotide primers might hybridize to nontarget sequences. This additional specificity made it possible to amplify segments of the human genome containing important disease-related allelic variations that alter defined enzyme restriction sites and simplify determi-

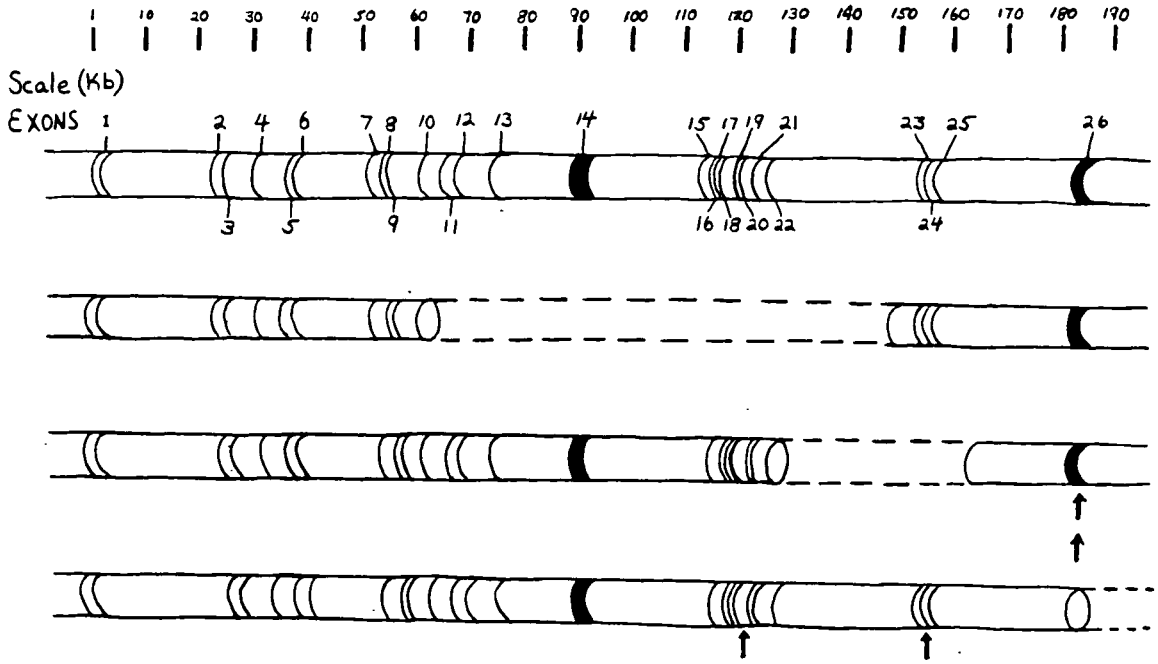



**Figure 8.13** The factor VIII gene, one of the largest genes cloned to date (186 kb), includes almost 0.1% of the entire X chromosome. The unusually large exon 14 (3.1 kb) encodes the entire B domain of factor VIII, which is removed from the protein during proteolytic activation by thrombin. In addition to the intact structure (top), diagrams show locations of partial deletions (dashed lines) and single base mutations (arrows) found in individual patients. Two single base mutations at exon 26 occurred in the same codon. One led to production of a truncated protein in a patient with severe hemophilia, the other to a single amino acid substitution associated with mild disease. *Reproduced from Vehar GA, Lawn RM: The cloning of factor VIII and the genetics of hemophilia A. Hosp Pract, May 15, 1986;21:5, with permission.*

nation of alleles by restriction enzymatic digestion. The higher-temperature *Taq* polymerization procedure with its additional specificity could be applied to a number of other restriction site polymorphisms, such as those in cystic fibrosis and Duchenne's muscular dystrophy. The speed, ease, and relatively low cost of this approach appear promising.

## Hemophilia B

Hemophilia B (Christmas disease) is an inherited, recessive sex-linked clotting disorder that is caused by a defect in clotting factor IX. It occurs in males at a frequency of about 1 in 30,000. As in hemophilia A, individual patients vary in the severity of clinical symptoms and in their clotting activity. Some heterogeneity in individual molecular factor IX defects is undoubtedly present. The initial characterization of the normal factor IX gene provided the means of studying hemophilia B.[81] It was observed that approximately 40% of hemophilia B patients have a normal plasma concentration of factor IX[82] and probably have point mutations in the coding sequence producing a single amino acid changes.[83] A few patients produce antibodies to the factor IX injected during replacement therapy, and in these instances the defect appears to be secondary to large deletions in the factor IX gene.[84] The remaining 60% of individuals with hemophilia B have either markedly reduced or

undetectable factor IX levels. Using molecular cloning techniques similar to those described for hemophilia A, a number of point mutations have been described in hemophilia B. In one instance, this was done by using an oligonucleotide probe that detected a single G → T point mutation within the obligatory GT of a donor splice junction.[85] The same technique was capable of detecting the mutant allele expression in an asymptomatic heterozygote.

Using the cloned factor IX gene transfected into mammalian cells as an expression vector, factor IX has been produced by several groups.[86,87] The cell lines transfected were shown to produce small amounts of active factor IX.

### *Carrier Detection*

Three RFLPs have been defined within the factor IX gene that allow inheritance of specific alleles to be determined.[88,89] This allows carrier detection and prenatal diagnosis with a very high degree of certainty. As new studies have appeared, additional probes and restriction fragments have been identified that will also prove useful in kindred and carrier analysis.[90,91]

### *Factor IX Locus in Hemophilia B Inhibitors*

As structural and clinical knowledge concerning the genes and gene products in hemophilia A and hemophilia B increased, it appeared that large gene deletions were often associated with eventual production of antibodies against the normal factors given periodically during therapy. This has been directly assessed in the case of the factor IX locus in nine hemophilia B patients who produced anti–factor IX inhibitors.[92] Two of the inhibitor patients had deletions of the entire factor IX gene. Two other inhibitor patients who were brothers had presumably identical complex rearrangements of the factor IX gene involving two separate deletions. Five other hemophilia B inhibitor patients appeared to have a structurally intact factor IX gene. Originally, Giannelli et al.[84] had proposed that hemophilia B patients produced antibodies to factor IX because of their lack of immune tolerance to deletional epitopes in the autologous factor IX mutants. This could be due either to a lack of expression of any or a significant part of the factor IX antigens or to synthesis of an altered factor IX lacking certain immunodominant antigenic epitopes. In the report by Matthews et al.,[92] several noninhibitor patients did not produce any measurable plasma factor IX. It is possible that such patients synthesize minute amounts of factor IX that are undectable in conventional assays. Alternatively, these patients may have lost the ability to synthesize factor IX after the development of actual tolerance. In summary, of 14 hemophilia B inhibitor patients reported, 8 had a detectable deletion of the factor IX gene; in contrast, only 2 of 19 hemophilia A inhibitor patients studied had a detected gene deletion.[84,92–94]

## Von Willebrand Disease

The general pattern of absence of gene product and correlation with development of reactive immune responses has also been examined in patients with von Willebrand disease who develop alloantibodies.[95–97] This disorder is associated with functional deficiency of a glycoprotein composed of 250-kd subunits essential for platelet adhesion to sites of vascular damage. As in the case of factors VIII and IX, the sequence of von Willebrand factor has been determined and its cDNA cloned.[98,99] In the study reported by Shelton-Inloes et al.[95] two unrelated patients

with severe recessive von Willebrand disease and alloantibodies to von Willebrand factor were found to have large deletions in the von Willebrand gene. By contrast, 19 patients with autosomal dominant type I von Willebrand disease and 17 additional patients with recessive von Willebrand disease did not have detectable deletions, and none of these 36 patients showed antibodies to von Willebrand factor. These findings tend to support the idea that large gene deletions are associated with an increased tendency to produce antibody to the deficient clotting factor. Similar observations have been reported by Ngo et al.,[97] who found alloantibodies only in kindreds with homozygous rather than heterozygous deletions. This particular point is of special interest because the general interplay between specific alterations in gene structure and exposure to the exogenous gene product by transfusion and the host immune response is still not fully understood. Despite the occurrence of a gene deletion, some individuals might be unable to recognize the corresponding protein antigens as foreign—even though these same antigens are not produced endogenously—because of possible inherited human leukocyte antigen (HLA)-linked features of their immune response. Eventually, precise definition of the epitope specificities of these interesting alloantibodies may help to unravel the puzzle.

## Other Clotting Factors

A number of other coagulation factors have been cloned and sequenced. Among them, human coagulation factor V is a high molecular weight plasma glycoprotein that is required for rapid thrombin formation and normal clotting.[100] This protein circulates in the blood as a large single polypeptide chain (330 kd) with little or no coagulant activity. During blood clotting, factor V is converted to factor Va by thrombin through a process of limited proteolysis; this exposes available binding sites for factor Xa and prothrombin. Factor V itself is composed of a 110-kd heavy chain and 76-kd light chain. The two chains are held together by calcium ions. The remainder of the factor V molecule is released as a large connecting fragment rich in carbohydrate. Factor V binds to cell surfaces and negatively charged phospholipid surfaces through the light chain and increases by about 10,000-fold the rate of prothrombin activation. Factor V is readily inactivated by activated protein C.

Human factor V was cloned from a phage λgt11 expression library using an affinity-purified antibody to factor V.[101] Clones were also identified using hybridization to an oligonucleotide complementary to the DNA coding for the amino acid sequence Met-Tyr-Glu-Gln-Glu-Trp-Val present in the light chain of human factor V. The isolated clones that contained the largest cDNA insert had 2970 nucleotides and coded for 938 amino acids. This region included 651 amino acids from the carboxyl terminus of the factor V light chain and 287 amino acids that were part of the connecting region of the protein. The amino acid sequence of the light chain of factor V is 40% homologous with the carboxyl-terminal fragment of human factor VIII. These fragments have a similar domain structure, which includes a single ceruloplasmin-related domain followed by two C domains. These similarities suggest that factor V, factor VIII, and ceruloplasmin are a family of related proteins that arose during evolution through gene duplication of the A domain.[102,103]

Factor XII, or Hageman factor, has also been successfully cloned and sequenced using a human cDNA library and mixtures of synthetic oligonucleotides.[104] Structural comparison showed extensive sequence identity with tissue-type plasminogen

activator as well as with fibronectin. Factor XIII, the clotting factor essential for covalent stabilization of the fibrin clot, has been cloned and sequenced[105] from a human placental λgt11 cDNA library. The gene for factor XIII α chain mapped uniquely to chromosome 6. Northern blot analyses clearly demonstrated mRNA for factor XIII in human placental tissues as well as several monocyte-like cell lines. The availability of cDNA probes for clotting factors such as XIII should provide an invaluable tool for examination of their role in body homeostasis.

## Gene Rearrangements in Hematological Disorders

Malignant conditions arising in cells of the hematopoietic system initially were classified on the basis of a histological and cytological system.[106] Later, with the rapid development of expression of various B or T cell lineage markers, an immunological classification emerged.[107–110] Subsequent analysis of immunoglobulin and T cell receptor genes has provided an additional dimension for classification and insight into the cellular origins of specific types of leukemias and lymphomas. Much of our current understanding of abnormal hematopoietic differentiation and expression naturally arose from careful studies of normal immunoglobulin[111–116] and later T cell receptor gene expression. New information in this area continues to emerge, particularly in the case of the various types or families of T cell receptors.

### B Cell Gene Rearrangements

Studies of normal pre-B cells maturing to immunoglobin (Ig)-bearing B cells have shown recombination of the light chain variable ($V_L$) and joining ($J_L$) segments prior to or concomitant with eventual integration with light chain constant regions ($C_L$).[117,118] Since both κ and λ light chain immunoglobulins are used in approximately 60% and 40% of human B cells, respectively, an ordered rearrangement of human Ig light-chain genes occurs in which κ chain rearrangements precede those of λ chains.[117] This was demonstrated by study of highly purified λ-bearing B cells from normal subjects. It was found that over 95% of the collective κ genes in the λ B cells were no longer in their germ-line form; the majority were deleted and the remainder were present but in the rearranged state.

With the knowledge of normal Ig gene rearrangement sequences, this approach was applied to an interesting group of patients with B cell leukemia, which is generally regarded as a stage in early lymphoid development arrested before active Ig synthesis is initiated.[118,119] It was found that although most of the pre–B cell leukemias had not acquired the capacity to synthesize detectable cytoplasmic immunoglobulin, they had undergone Ig gene rearrangements that appeared to commit them to eventual B cell development. Moreover, the patterns of Ig gene rearrangement in the pre-B leukemic cells indicated the schedule for Ig gene rearrangements.[120,121] This showed that heavy-chain (μ) genes were first, followed by κ and then λ light chains.

In the original work eight patients were studied. Leukemic cells showed no sheep cell rosetting behavior nor any evidence of surface Ig. All patients showed acute lymphocytic leukemia (ALL)-associated antigen[118] and six patients were positive for Ia antigen. In this study genomic DNA was obtained from leukemic cells and compared to normal germ-line DNA harvested from the patients' fibroblast or normal-remission leukocytes. $C_\mu$, $C_\kappa$, and $C_\lambda$ DNA probes were used to detect Ig gene rearrangements. Representative results for these informative cases are shown

in Figure 8.14. The leukemia patients could thus be placed in a scheme of development from primitive stem cell through pre–B cell leukemia to B cell leukemia, depending on the succession of Ig gene rearrangements and deletions. It was thus possible to devise a tentative scheme of cell development from stem cell to B cell leukemia. The basis for such a model is shown in Figure 8.15. In this scheme a stem cell would be expected to show all of its immunoglobulin genes in the germ-line configuration and to initiate immunoglobulin gene rearrangements by joining $V_H$, $D_H$, and $J_H$ segments to provide the complete nucleotide units toward forming a $\mu$ chain. Because $\kappa$ light chain genes precede $\lambda$ light chain rearrangements, the two $C_\kappa$ alleles could undergo a functional, aberrant, or deletional rearrangement. Cells containing rearranged $\mu$ and $\kappa$ alleles should then be capable of maturation to B cells bearing surface Ig. In like manner, $\lambda$ genes could rearrange to produce either complete surface Ig or aberrant patterns, thus preventing further progression toward full development.

On the basis of immunoglobulin gene rearrangements, patients with non-T, non-B leukemias showed malignant transformation of cells that were originally committed to B cell differentiation. This was a major step toward insight into cell differentiation pathways in this group of patients, because previously only 15% to 20% of them had been classified as pre-B on the basis of cytoplasmic $\mu$ chain staining.[122,123] Five of the eight leukemic patients studied by Korsmeyer et al.[118] had clear-cut Ig gene rearrangements but no detectable immunoglobulin protein product.

## Acute Lymphocytic Leukemia

This general approach was next applied to a much larger group of 37 cases of acute lymphocytic leukemia.[121] All 12 cases of the T cell type showed germ-line $\kappa$ and $\lambda$ Ig gene patterns, and 11 of 12 showed germ-line heavy chain genes. By contrast, all 25 cases in the non-T, non-B classification, lacking definitive T cell markers or surface Ig, showed rearranged Ig genes. This indicated that they represented precursor cells already committed to the B cell lineage. When the patterns of immunoglobin gene rearrangements in this large group of patients were correlated with the results of cell surface marker studies, it appeared that expression of HLA-DR preceded reactivity with BA-1, a monoclonal antibody defining B cell populations.[122] Furthermore, BA-1 reactivity occurred before positive antibody reactions were detected with the common acute lymphoblastic leukemia antigen (CALLA).[123] The parallel analysis of cell surface markers and Ig rearrangements is shown diagrammatically in Figure 8.16. It was postulated that lack of Ig production by the majority of leukemic B cell precursors might be due to a variety of factors involved in B cell differentiation, including mistaken rearrangements or deletion of all $D_H$ gene families.

## Malignant Lymphomas

Gene analytic techniques quickly proved extremely useful in the analysis of malignant lymphomas. Since it had been recognized from cell marker studies that most non-Hodgkin's lymphomas were probably of B cell origin,[124] it was useful to explore this clonal B cell expression in terms of Ig gene rearrangement and expression patterns. In an analysis of patients with various lymphoproliferative diseases, the abnormalities found by cell surface staining and flow cytometry

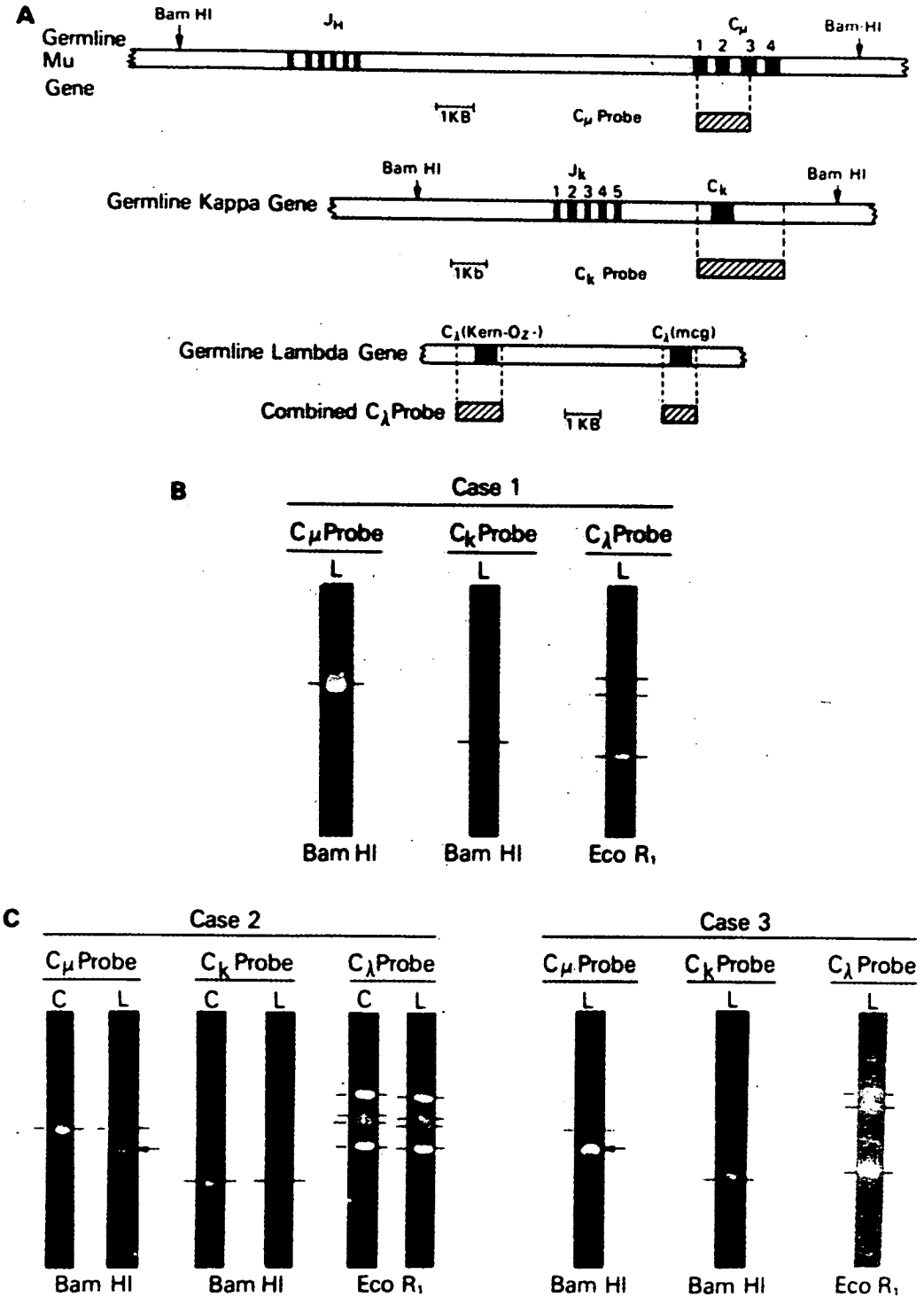



**Figure 8.14** (A) Human $C_\mu$, $C_\kappa$, and $C_\lambda$ DNA probes utilized to detect germ-line and rearranged immunoglobulin genes. In order to demonstrate $\mu$ chain gene rearrangements, the *Bam*HI digests of the leukemic lymphocytes were hybridized with a 1.5-kb embryonic *Eco*RI probe. The *Bam*HI digests were also hybridized to a probe consisting of a 2.5-kb embryonic *Eco*RI $C_\mu$-containing fragment. The combined $C_\lambda$ probe used to detect $\lambda$ light chain gene rearrangements in *Eco*RI digests consisted of a 1.2-kb embryonic *Bam*HI-*Eco*RI fragment containing the Mcg $C_\lambda$ gene. This combined $C_\lambda$ probe is capable of cross-hybridizing to all of the nonallelic $C_\lambda$ genes. (B) Case 1: a germ-line pattern for $\mu$, $\kappa$, and $\gamma$ genes. The $C_\lambda$-containing gene fragment in the leukemic lymphocytes (L) of case 1 was a single band

showed a high degree of correlation with monoclonal Ig gene rearrangements as detected by Southern blot analysis.[125] These patients included individuals with non-Hodgkin's lymphoma and a few with hairy-cell leukemia, chronic lymphocytic leukemia, lymphosarcoma, and Hodgkin's disease. Earlier work had established the usefulness of this technique in studying human lymphoid neoplasms and in differentiating a malignant proliferative process from benign lymphoid hyperplasia.[126] In the past, monoclonality in lymphoid neoplasms had been identified by use of immunofluorescence or glucose-6-phosphate dehydrogenase markers.[127] Southern blot analysis of DNA from lymphoid tumors has proved to be of great usefulness in analyzing lymphomas or lymphoid neoplasms without a predominant cell immunotype. Mixing experiments to determine the level of sensitivity of this approach indicated that when genomic DNA from a T cell with germ-line Ig genes or from a polyclonal source of B cells such as normal human tonsils was admixed in various ratios with DNA from a monoclonal B cell line showing clear-cut Ig gene rearrangements, the clonal rearrangement was detectable when only 10% of the DNA hybridized contained rearranged Ig genes.[126] The great precision of this approach is illustrated in Figure 8.17 from the study by Arnold et al.[126] These studies also showed the value of Southern blotting in establishing the diagnosis of lymphoma in cancers of uncertain types, such as case 1 in Figure 8.17.

Immunoglobulin gene rearrangements in leukemias and lymphomas of the B cell series at all stages of maturation are useful because they serve as markers for individual tumors. Their identification after many courses of chemotherapy or radiation may indicate tumor persistence or of early recurrence. With other commonly used methods it is often very difficult or impossible to detect malignant cells present in small proportions in various body fluids or tissue specimens. The use of in situ or extracted DNA hybridization procedures capable of detecting 5% to 10% Ig gene rearrangements adds another dimension of sensitivity to diagnosis.

Application of Southern blotting to DNA from cells of patients with diffuse histiocytic lymphomas using Ig gene probes showed heavy and light chain gene rearrangements in a majority, thus placing these tumors in the B cell lineage.[128] The absence of Ig gene rearrangement in one patient indicated that the tumor might represent a true histiocyte or uncommitted stem cell.

identical to the 19.3-kb *Bam*HI germ-line fragment displayed by the non–B cell sources of DNA from 30 individuals (indicated by lines extending from the gel). Likewise, the $C_\kappa$ probe revealed the κ genes to be in their germ-line configuration in a 12.0-kb *Bam*HI fragment similar to other non–B cell sources of DNA (indicated by lines). Case 1 displayed a germ-line configurations of λ chain genes referred to as a "type I" pattern, consisting of an upper band of 16.0 kb (actually a close doublet of 16.2 and 15.8 kb), a 14.0-kb fragment, and a lower 8.0-kb fragment. (C) Cases 2 and 3: μ genes rearranged, germ-line light chain genes. Peripheral leukocytes obtained during a clinical remission served as a control (C) for the germ-line pattern of immunoglobulin genes in case 2. The leukemic lymphocytes (L) of cases 2 and 3 showed a rearranged μ gene (arrow) with apparent loss of the other $C_\mu$ allele (the expected germ-line positions of the gene-containing fragments are indicated by lines extending from the gels). The κ and λ genes are in germ-line configurations. The type II germ-line configuration for $C_\lambda$ genes is displayed by case 2 and consists of an upper 18.0-kb fragment, the close doublet at 16.0 kb (16.2- and 15.8-kb fragments), and the 14.0-kb fragment. These two germ-line configurations of $C_\lambda$ genes in humans result from inherited differences in the location of *Eco*RI sites surrounding the $C_\lambda$ genes. *Reproduced from Korsmeyer et al,*[118] *with permission.*

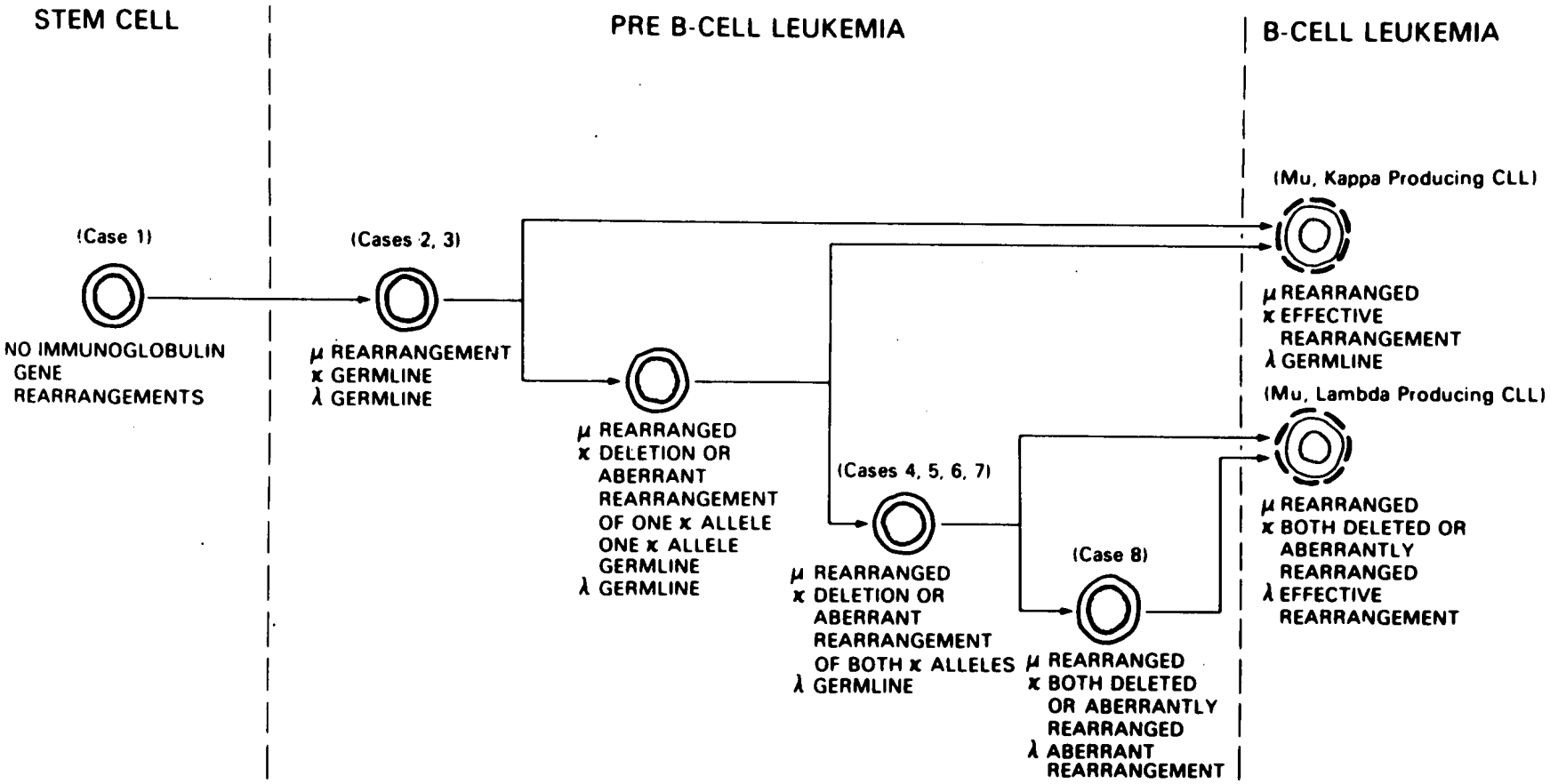



**Figure 8.15** Patterns of immunoglobulin gene rearrangement in pre–B cell leukemia. In this hypothetical scheme of differentiation, a stem cell or B cell lymphoid precursor with germ-line immunoglobulin genes would initiate immunoglobulin gene activity by rearranging the μ gene (a pattern seen in two cases). In general, κ gene rearrangements would follow, and, if effective, the cell could mature into a B cell bearing surface μ and κ chains while retaining germ-line λ genes. If all the κ gene recombinations were aberrant or deletional, attempts at effective λ gene rearrangement could follow. *Reproduced from Korsmeyer et al,*[118] *with permission.*

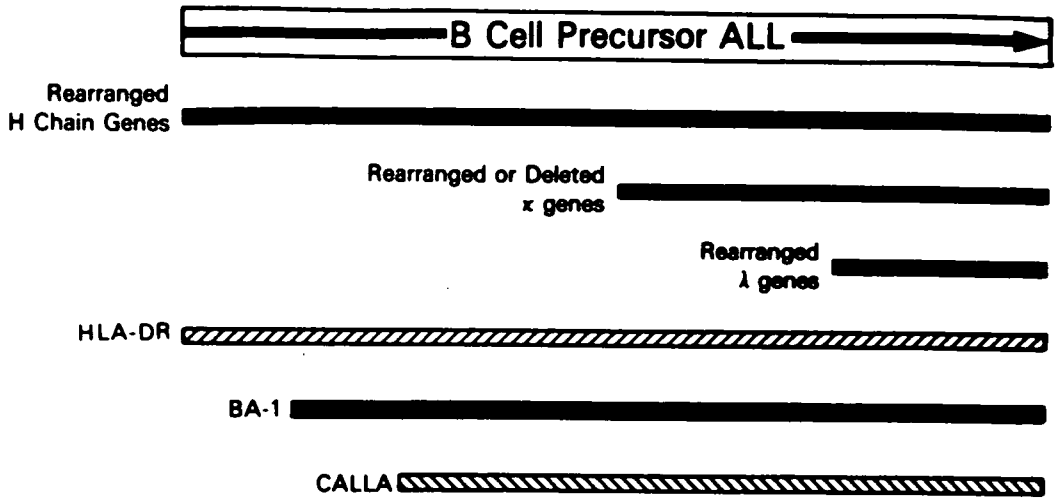



Figure 8.16 A coordinated sequence of Ig gene rearrangements and B cell surface antigen expression. Comparisons here suggest that rearranged H chain genes and Ia-DR molecules precede the appearance of BA-1 reactivity and CALLA. Cases with recombined light chain genes always had rearranged heavy chain genes and usually displayed all three of the surface antigens. *Reproduced from Korsmeyer et al.*[121] *with permission.*

## Linkage Infidelity and Gene Rearrangements

There is evidence that Ig gene rearrangement may not always be restricted to leukemias or lymphomas of B lineage but may also occur in phenotypically defined acute myeloid leukemias[129] and acute T cell lymphocytic leukemia.[130] In some instances even biphenotypic expression of both myeloid and immunological markers could indicate that leukemic transformation occurred in an immature stem cell capable of differentiating into two distinct lineages.[131,132] A simple theory of irreversible lineage commitment to one differentiation line cannot explain apparent instances of anomalous myeloid or lymphoid markers occurring outside their expected lineages.[132,133] These phenomena, which include the identification of nuclear terminal deoxyribonucleotidyltransferase (TdT) in leukemic myeloblasts[134] and the inappropriate rearrangement of Ig or T cell receptor genes in lymphoid or myeloid lineage cells,[135–137] are now referred to under the collective term lineage infidelity.[138] It could be that this anomalous situation reflects normal stages of cell differentiation that are frozen or somehow selected by the leukemic transformation. Therefore lineage infidelity has also been called lineage promiscuity to indicate a physiological rather than a dyssynchronous process.[133]

This subject has been investigated by Seremetis et al.[139] with the idea that gene rearrangement and TdT expression might be associated and represent abortive activation of lymphoid line initiation before irreversible commitment. Results of this study indicated a high frequency (>60%) of Ig and/or Tβ gene rearrangements in a subset of acute myelogenous leukemia patients selected on the basis of expression of the lymphoid lineage TdT marker. These findings appear to indicate that TdT must be involved in both Ig and T cell receptor rearrangements and that true linkage infidelity is not present. Alternatively, leukemias with TdT expression and both Ig and T cell receptor rearrangements may represent a clonal expansion of a normal hematopoietic precursor that is scarce in normal hematopoietic differentiation. Furthermore, these findings raise a note of caution concerning the use of Southern

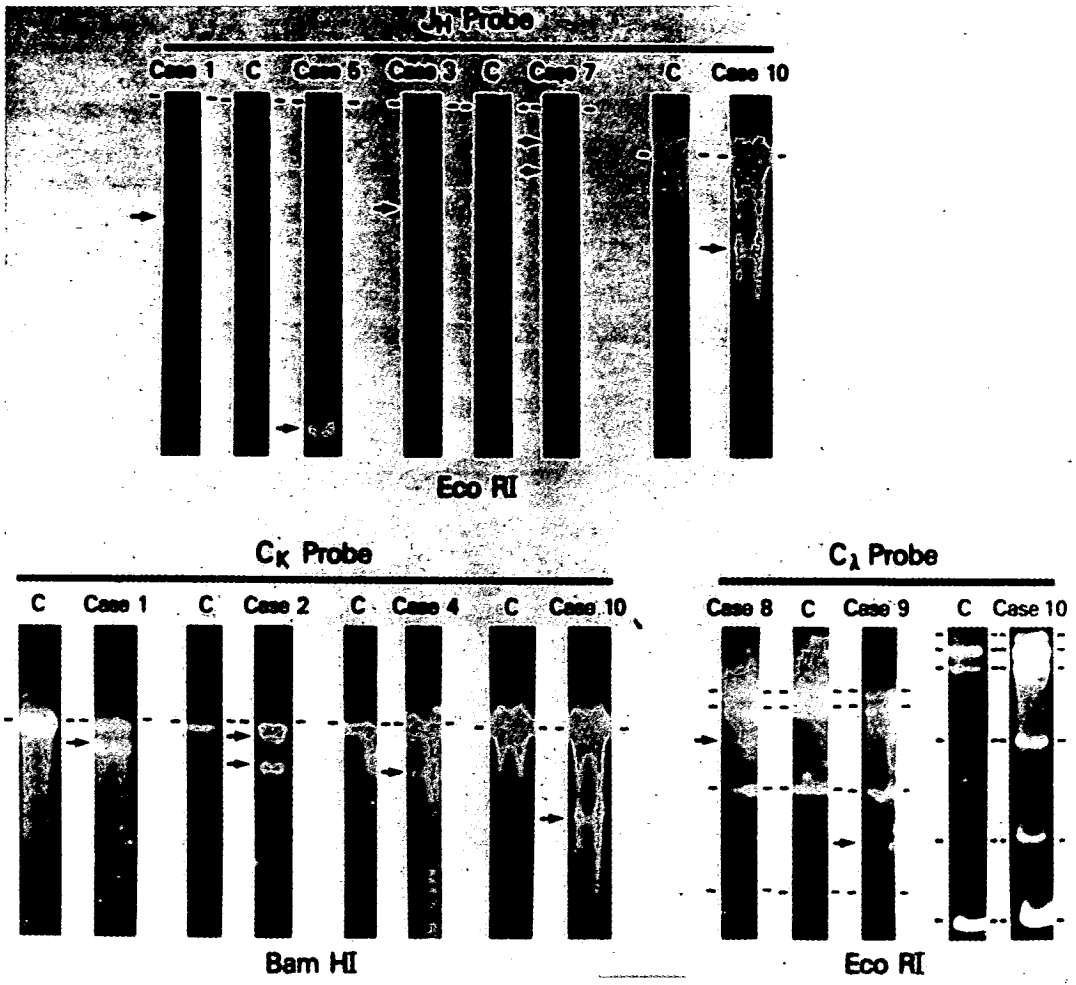



Figure 8.17 Representative Southern blot analyses of immunoglobulin genes from lymphoproliferative tissues and nonlymphoid control DNA(C) with germ-line immunoglobulin gene configurations. The dash marks indicate the germ-line position of the probed gene within the particular endonuclease digests and the arrows show the position of the rearranged alleles. Case 1, undifferentiated malignant tumor; case 5, follicular and diffuse mixed-cell lymphoma; case 3, diffuse large cell lymphoma; case 7, follicular small-cleaved cell lymphoma; case 10, atypical follicular hyperplasia; case 2, follicular large cell lymphoma; case 4, diffuse mixed-cell lymphoma; case 8, follicular small-cleaved cell lymphoma; case 9, diffuse large cell lymphoma. Note that $C_g$ in case 10 is not rearranged, since the extra bands seen represent a restriction fragment length polymorphism shared by the lymph node DNA and the same patient's nonlymphoid control DNA. No such polymorphisms exist for the human $J_H$ or $C_k$ loci, so placental DNA is used as a control in the other cases. *Reproduced from Arnold et al,*[126] *with permission.*

blot analysis and Ig as well as Tβ gene rearrangements for absolute identification of clonal lymphoid populations; further comparative analyses are clearly in order.

## Premalignant Conditions

Immunoglobulin gene rearrangement analysis has been applied to several human disorders thought to be premalignant or associated with eventual neoplastic transformation. Angioimmunoblastic lymphadenopathy was studied by DNA Southern blot analysis.[140] Lymph nodes and peripheral blood from these patients showed

clones of lymphoid cells with either Ig or T cell receptor gene rearrangements, which in some instances regressed during the course of disease. These findings were of particular interest because the proliferating lymphoid clones did not always appear to be fixed in a rearranged gene phenotype but actually disappeared during the course of disease in several patients. A lymph node from one patient was involved by immunoblastic lymphoma and showed an additional gene rearrangement pattern not observed in premalignant samples from the same individual. These studies indicate that presence of one or more rearranged non–germ line bands in Southern blots of lymphoid tissues from patients with angioimmunoblastic lymphadenopathy do not always indicate malignancy. They are also pertinent to the problem of B cell clonal proliferation in patients with immune deficiency or profound immunosuppression, as in the case of marrow or cardiac transplantation.[141,142]

## Sjögren's Syndrome and Immunoglobulin Gene Rearrangement

Immunoglobulin gene rearrangements have been uniformly detected in benign lymphoepithelial lesions found in association with Sjögren's syndrome.[143] Southern blot hybridization techniques were used to examine DNA from salivary gland tissues from eight patients with benign lymphoepithelial lesions. Ten samples from the eight patients all revealed rearrangement of both heavy-chain and light-chain Ig genes. One patient had two benign lymphoepithelial lesions removed 2 years apart. The heavy-chain rearrangements and the rearrangements detected with κ light chain genes in the two separate lesions were entirely different. Because detection of a gene rearrangement has been regarded as strong evidence for neoplastic lymphoid proliferation, these findings were of considerable interest. Patients with Sjögren's syndrome and lymphoepithelial tumors are generally regarded as having increased susceptibility to the development of non-Hodgkin's lymphoma. Although the clonal expansions in this study may reflect premalignant changes, this appears unlikely in view of their clinical regression. Another possible explanation is that the clonal expansions reflect a generalized defect in immune regulation that is intrinsic to this autoimmune disorder. Parallel observations on normal human tissues involved in polyclonal B cell activation or a normal immune response would be useful as a baseline for evaluating the significance of Ig gene rearrangements in disorders like Sjögren's syndrome, in which a small but significant proportion of patients eventually develop a lymphoid malignant process.

## T Cell Receptor Genes and Their Rearrangements

In parallel with the work on Ig gene rearrangements and interpretations of clonality and potential malignancy, the same approach was applied to the analysis of T cell receptor genes in human leukemias and other lymphoproliferative disorders. One of the earliest aspects examined was T cell receptor β chain rearrangement.[144–147] Initially, virtually all T cell leukemias and most T cell lymphomas examined showed β chain gene rearrangement for the T cell receptor. As in the cases of Ig genes and Sjögren's syndrome, some anomalous results were recorded, including T cell receptor rearrangements in some chronic lymphocytic leukemias and immunoblastic lymphomas. A schematic diagram of the T cell receptor β chain gene is shown in Figure 8.18. Clear-cut rearrangements are seen in Figure 8.19. Beta chain rearrangements were readily detected in a patient with human lymphotropic virus type

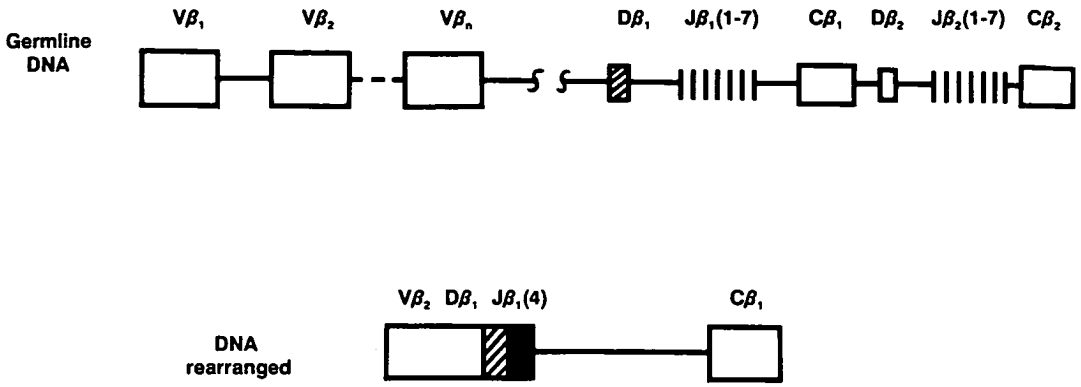



**Figure 8.18** T cell receptor β chain gene in germ line (above) and reassembled (below). Variable ($V_\beta$), diversity ($D_\beta$), joining ($J_\beta$), and constant ($C_\beta$) segments are rearranged, followed by primary mRNA transcription from the rearranged segments below.

I (HTLV-1), as well as in a patient with cutaneous T cell lymphoma of the Sézary type.[147] A patient with T gamma lymphocytosis, a disorder that has not yet been defined in terms of a clonal T cell neoplasm, was studied by Bertness et al.,[146] who found no T cell receptor rearrangements. Moreover, the method proved useful in patients who were thought to develop a separate or new malignant process during long-term treatment and follow-up. In such instances, changes in T cell gene rearrangement patterns clearly identified emergence of a second or new malignant process.[146] Waldmann et al.[147] studied a patient with $T_8$ lymphocytosis and granulocytopenia and anemia (a syndrome characterized as a unique clinical entity by several groups[148–150]) and found definite T cell β chain rearrangement, making it likely that a malignant clonal expansion of cells was present.

## T Cell Receptor Rearrangement Order

As in the case of Ig genes, T cell receptor genes undergo rearrangement in an ordered progression. The first to rearrange is the T cell γ chain gene, followed by T cell β chain gene and finally α chain gene rearrangement.[151] Studies of a number of

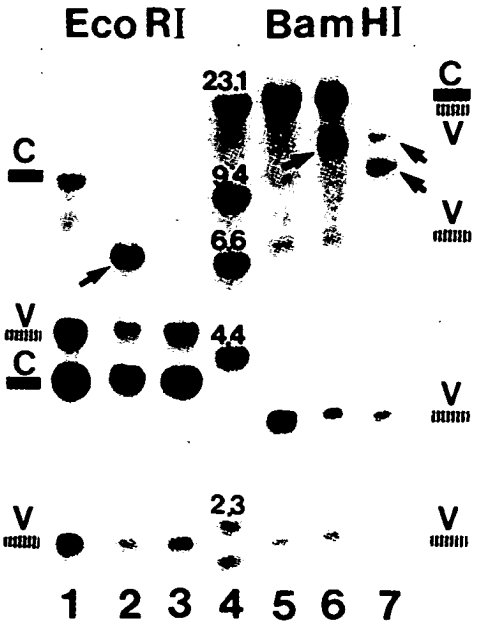


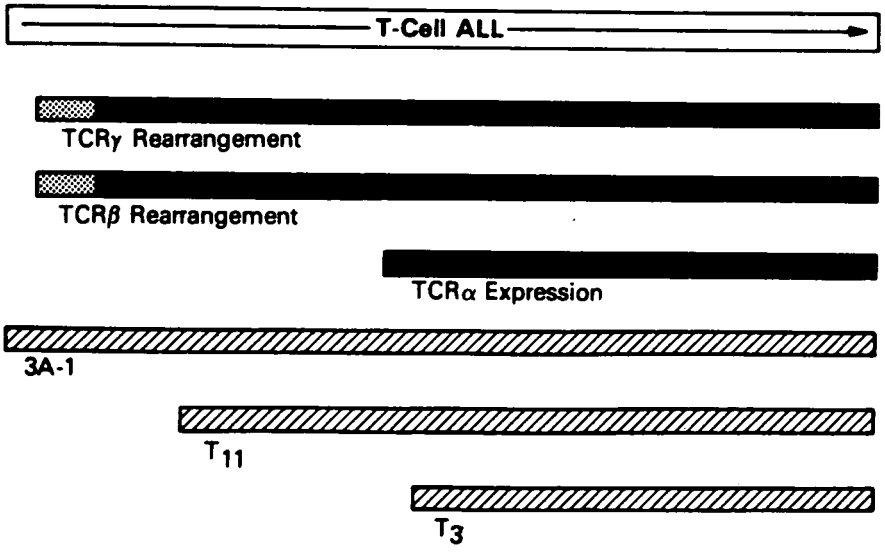

**Figure 8.19** Rearranged DNA fragments bearing the gene (arrows) for the constant region of the β chain of the T cell receptor in a patient with T4 chronic lymphocytic leukemia (lanes 2 and 6) and in a patient with adult T cell (T4) lymphoma (lanes 3 and 7). The germ-line positions of the constant (C) and variable (V) β chain bands after *Eco*RI digestion (lanes 1 to 3) are indicated at the left and illustrated in DNA prepared from a thymoma (lane 1); the positions after *Bam*HI digestion (lanes 5 to 7) are indicated at the right. *Reproduced from Aisenberg et al.*[145] *with permission.*

leukemic cell lines have shown $T_8$ chain genes rearrangement in leukemic B cells as well as T cells.[152] Use of the T cell receptor α chain genes as evidence of rearrangement has not proved practical, primarily because of the dispersed organization of the $J_\alpha$ regions in the α chain locus.[153]

In a further assessment of the specificity of T cell receptor rearrangement for lineage of tumor cells in certain groups, T cell β and γ chain rearrangements were studied in 100 consecutive B cell lymphomas and B cell chronic lymphocytic leukemias by Aisenberg et al.[154] In only two cases were T cell receptor rearrangements detected, and γ chain rearrangement was not recorded in any of the patients. This is in sharp contrast to the findings in non-T, non-B acute lymphocytic leukemias, where almost half the patients examined showed rearrangement of one allele of the T γ chain and a significant proportion showed rearrangement of one T β allele.[155]

The question of T cell receptor expression and rearrangement in acute pre–B cell lymphoblastic leukemias was also addressed by examination of α, β, and γ T cell receptor gene activation in a relatively broad spectrum of patients with this disorder.[156] A developmental hierarchy of T cell receptor gene activation was suggested, in parallel with studies of Ig gene activation and cell surface marker studies. A coordinated sequence of gene expression based on these observations is shown in Figure 8.20. As in previous work, there was some evidence of what was termed lineage spillover, in that 10% of patients with acute T cell lymphocytic leukemia showed immunoglobulin heavy-chain gene rearrangements. Likewise, patients characterized as being pre-B showed a surprising amount (45%) of T γ chain receptor rearrangement as well as T β chain rearrangement (20%). Whether these findings are related to so-called lineage fidelity remains to be determined. As pointed out by Korsmeyer,[157] the final gold standards of lineage determination remain to be established, since lineage spillover or lineage promiscuity appears to

**Figure 8.20** Proposed coordinated sequence of T cell receptor (TCR) rearrangement and expression plus cell surface antigen expression in T cell acute lymphoblastic leukemia (T ALL). 3A-1 appears to precede TCR gene rearrangement. The λ and β TCRs rearrange early in development, but in no fixed order. Most T ALLs are mature enough to have rearranged both λ and β TCRs and approximately one-half express the α TCR locus. The T11 molecule follows 3A-1 and T3 appears around the time of α TCR expression. *Reproduced from Felix et al,[156] with permission.*



occur in several neoplasms, including pre-B acute lymphocytic leukemia and some cases of acute myelocytic leukemia. Basic genomic recombination signals (heptamer CACAGTG-spacer-nonamer [ACAAAAACC] that flank these gene segments are very similar in both Ig and T cell receptor genes and may be the recognition region for a recombinase enzyme capable of rearranging either Ig or T cell receptor genes.[157,158] Such a recombinase might help to explain the crossover of both Ig and T cell receptor gene rearrangements into the opposite lineage, perhaps representing vestigial elements of an early decision-making process that occurs before final commitment to B or T cell lineage differentiation.

## Clinical Applications

One question about Ig or T cell receptor gene rearrangement analyses is whether these techniques can be helpful to the clinician who treats patients. In some patients clinical utility has already been demonstrated, since acute lymphocytic leukemias or lymphomas have been shown to display different profiles of rearrangement when followed sequentially over a period of time. In some instances, data from serial gene rearrangement studies appear to indicate that some tumors are biclonal or have evolved an entirely new neoplasm.[159,160] Whether such transformations actually reflect emergence of new tumors or evolution of subclones of a single malignant progenitor is still problematic. In many of these instances parallel analysis of multiple clonal markers can be interpreted merely to represent clonal or original tumor heterogeneity.[160] It is conceivable that mature B cell neoplasms could reactivate some sort of recombinase mechanism to result in these additional rearrangement modifications. In a parallel way, idiotypic variation has been observed to result from straightforward somatic mutations.[161]

## Chromosomal Translocation

The relationship of specific chromosomal translocations to certain well-defined neoplasms has been discussed in Chapter 5 with respect to Burkitt's lymphoma and to the Philadelphia chromosome and chronic myelocytic leukemia. It is of considerable interest that in lymphoid neoplasms, the same gene segments that normally rearrange to produce antigen receptor molecules such as surface Ig in B cells or the T cell receptor in T cells also appear to be involved in these interchromosomal translocations. In Burkitt's lymphoma the chromosomal break point occurs at the Ig gene loci (8q 24.13 and 14q 32.33). Chromosomal break points at 14q32 occur repeatedly in other mature B cell tumors besides Burkitt's lymphoma.[162] Other studies now indicate that the most common lymphoma translocation is typical of follicular lymphoma and puts a new B cell–associated gene from chromosome location 18q21 into 14q32. Although follicular lymphomas are phenotypically mature B cells, translocations appear to occur at the D-J rearrangement step very early in pre–B cell differentiation. When such break points in chromosomes are carefully analyzed, it appears that the major site is on chromosome 18. Thus, over 70% of t(14;18) translocations occur within a 2- to 3-kb major break point region; moreover, most of these break points occur in a cluster within a region of 150 base pairs.[161,162] Such break point clustering must have a functional role. It is of interest that these occur in a 3′ exon of a B cell–associated gene, making a fusion transcript with the Ig $J_H$ region. In summary, it now appears that malignancy-associated translocations in T cells are found classically at T cell re-

TABLE 8.2 Uses of Gene Rearrangement in Hematopathology

1. Distinguish clonal from polyclonal lymphoproliferations.
2. Determine the B or T cell identity of malignancies with admixed normal cells.
3. Determine the genetic lineage of neoplasms lacking definitive surface antigens.
4. Determine the developmental stage of early B or T cell precursors.
5. Identify unanticipated stages of development not appreciated within normal cell populations.
6. Tumor-specific markers to follow the natural history of neoplasms.
7. Search for minimal residual disease during clinical remission.
8. Molecularly mark chromosomal translocations and identify new transforming genes.

ceptor loci, whereas many of the B cell translocations, as in Burkitt's lymphoma, occur close to B cell receptor or Ig regions. A general summary of the use of gene rearrangement analysis in hematopathology is shown in Table 8.2.

## Gene Rearrangements and the Sometimes Fuzzy Interface with Benign Conditions

Gene rearrangement analysis to establish clonality of various conditions probably has yet to reach its maximal use in clinical medicine. One of the most useful applications thus far has been in the diagnosis of mycosis fungoides or other malignant conditions of the skin. In many cases, clinical evaluation of patients suspected of having mycosis fungoides has been extremely difficult, even with expert histological review of tissue sections. This problem has now been addressed using Southern blot analysis for clonal arrangements of the T cell β receptor.[163] In this study evidence for T cell β chain rearrangements was closely correlated with histologically unambiguous positive tissue identification. Moreover, clonal rearrangements were found in seven of nine lymph nodes from mycosis fungoides patients that had been considered to show only benign lymphadenopathy.[163]

This approach has been rapidly adopted by oncologists and dermatopathologists, and it now represents a fundamental example of application of recombinant methods to initial precise clinical evaluation of problem patients. The same approach has been applied to lymphomatoid papulosis, which appears to be a cutaneous neoplastic process with a relatively benign course.[164] Studies of biopsy tissues in these patients showed multiple patterns of T cell receptor β gene rearrangement even in a single patient. These findings support the concept that the disease represents a multiclonal malignant T cell neoplasm. Similarly, T cell receptor β gene rearrangement has now been reported in pagetoid reticulosis, another rare skin disorder of unknown cause.[165] Such findings again present strong evidence that this condition is a T cell neoplastic process.

## Monoclonal Cryoglobulinemia

Monoclonal cryoglobulinemia is a disease of unknown etiology with the clinical presentation of peripheral small vessel occlusions and arteritis, exacerbation by cold exposure, and the presence of distinct cryoglobulins in the serum. Several types of cryoglobulinemia have been described. Some patients produce monoclonal IgG, IgA, or IgM paraproteins that precipitate on cold exposure, whereas others have mixed cryoglobulins comprising a monoclonal IgM rheumatoid factor and

autologous polyclonal IgG. Monoclonal cryoglobulinemia is generally not regarded as a malignant process. Four patients with monoclonal IgG cryoglobulins were studied in detail by Perl et al.[166] Southern Blot analysis of DNA extracted from peripheral blood of four patients showed Ig gene rearrangements in three of the four. In addition, the c-*myc* gene locus was altered in genomic DNA from peripheral blood leukocytes in two of the patients. These findings illustrate the high sensitivity of this approach. In patients with cryoglobulinemia it is estimated that 0.5% to 2% of the total peripheral blood B lymphocytes may be engaged in production of the monoclonal protein. The cryoglobulin is detected because of the profound biological effects it has in the circulation and particularly in vulnerable peripheral vessels. This disorder is not regarded as a cancer, yet it is assumed that it must have a monoclonal origin because of the homogeneous monoclonal immunoglobulin product. Similar Ig *myc* heavy chain rearrangements have been reported in patients with mixed IgM-IgG cryoglobulinemias.[167] It remains to be seen whether Ig gene rearrangement alone can be used in such patients as a criterion of malignancy.

## Gene Rearrangement and Plasma Cell Myeloma

Because multiple myeloma clearly represents a malignant process, with production of osteolytic metastatic bone lesions and eventual overwhelming of the host, Ig gene rearrangement in the affected B cells is expected and has now been documented. No preferential use of any particular VH(V) region genes was found in the multiple myeloma patients studied.[168] A patient with both chronic lymphocytic leukemia (CLL) and multiple myeloma was studied to examine parallel B cell Ig gene rearrangements.[169] The CLL clonal character was IgM κ and the multiple myeloma IgA λ. Despite the different isotypes of the tumors, pretreatment studies of the heavy chain genes ($J_H$) showed a germ-line fragment and two identical rearrangements in blood and marrow. Both κ and λ light-chain genes showed rearrangement in the blood, suggesting involvement of peripheral blood lymphocytes by the myeloma. Analysis of the Ig genes after chemotherapy demonstrated no change in $J_H$ or $C_\kappa$ rearrangements, but the λ genes showed a germ-line configuration. These fascinating results suggested that both CLL and the plasma cell myeloma originated from the same B cell progenitors.

That myeloma may arise from a pluripotent stem cell is indicated by the finding of erythroid and megakaryocytic antigens on some myeloma cells and the demonstration of rearrangement of the T cell receptor γ genes in myeloma tumor cells with parallel rearrangement of Ig heavy-chain genes.[170]

Conventional search for Ig gene rearrangement in multiple myeloma, using comparative analysis of B cells from peripheral blood and bone marrow by Ig affinity and magnetized beads,[171] has clearly indicated uniform gene rearrangement in most patients studied. Of great interest would be analysis of patients with so-called benign monoclonal gammopathy, who display a monoclonal IgM serum component for years without developing clinical myeloma. Because such patients must harbor plasma cells with Ig genes rearranged to produce the monoclonal IgM component, studies of their marrow or peripheral blood B cells would be of great interest and might be predictive of subsequent development of malignancy. In this regard, some element of control or even autocrine regulation of plasma cell differentiation has been suggested for the lymphokine interleukin 6 (IL-6).[172,173] DNA rearrangement and constitutive expression of the IL-6 gene in a mouse plasma-

cytoma has been reported by Blankenstein et al.[174] The findings provided evidence that the IL-6 gene was expressed within the plasmacytoma and that the cell line actually produced IL-6 in the cell supernatants. Because IL-6 is a known growth factor for many types of immune cells, it may be related to the difference between benign monoclonal gammopathy and frank plasma cell myeloma.

### Molecular Gene Rearrangements in Hematopathology

As noted in the foregoing sections, development of molecular biological techniques to supplement available information on patients with various malignancies of the immune or lymphoid system has progressed rapidly. Gene rearrangement analysis can be performed on virtually any tissue, including lymph node biopsy specimens, bone marrow aspirates, peripheral blood, body fluids, and cell suspensions. Frozen tissue in cryostats is also suitable for gene arrangement analysis. The limitations of gene rearrangement study are that it does not directly identify which cells are clonal because a mixture of DNA from a tissue is analyzed; the sensitivity of the method is 1% to 5%, and the testing in the current stage is labor intensive. The method enhances rather than supplants careful histologic and immunohistologic as well as cell-sorting analyses.[175]

## Recombinant Products and Clinical Hematology

### Erythropoietin

Successful cloning of the gene for human erythropoietin[176,177] was a major positive achievement for clinical medicine. Methods used included initial purification, amino acid sequence analysis, and subsequent construction of oligonucleotide probes based on the determined sequence. A human cDNA library prepared in bacteriophage λ was screened and clones showing positive hybridization were isolated. The structure of the erythropoietin gene was then deduced using overlapping sequences obtained by this methodology. Once the primary sequence and exact protein structure of this important hematopoietic hormone were established, precise studies of its biological activity and its tissue sources and metabolism became possible. The story of erythropoietin is a remarkable tale of the application of a recombinant technology, with an extremely short interval until the product was directly applied to important human clinical needs and problems. For some time it was known that erythropoietin is produced principally by the kidney in adults and by the liver during fetal life. Circulating levels of the hormone are normally approximately 20 mU/ml,[178,179] and they increase substantially during conditions of tissue hypoxia. Damage to the kidney such as occurs in chronic renal failure results in anemia due primarily to a deficiency in erythropoietin production.[180,181]

#### *Cloning the Erythropoietin Gene*

The isolation and cloning of the human erythropoietin gene proved to be a problem because initially no ready source of mRNA was known. Furthermore, no cell lines producing large amounts of erythropoietin were available, and although the kidney had been identified as the most likely source, it was not clear initially whether induction of erythropoietin resulted from de novo synthesis or activation of an inactive precursor molecule. Thus the whole cloning strategy was based on obtaining some initial accurate sequence data in order to construct oligonucleotide

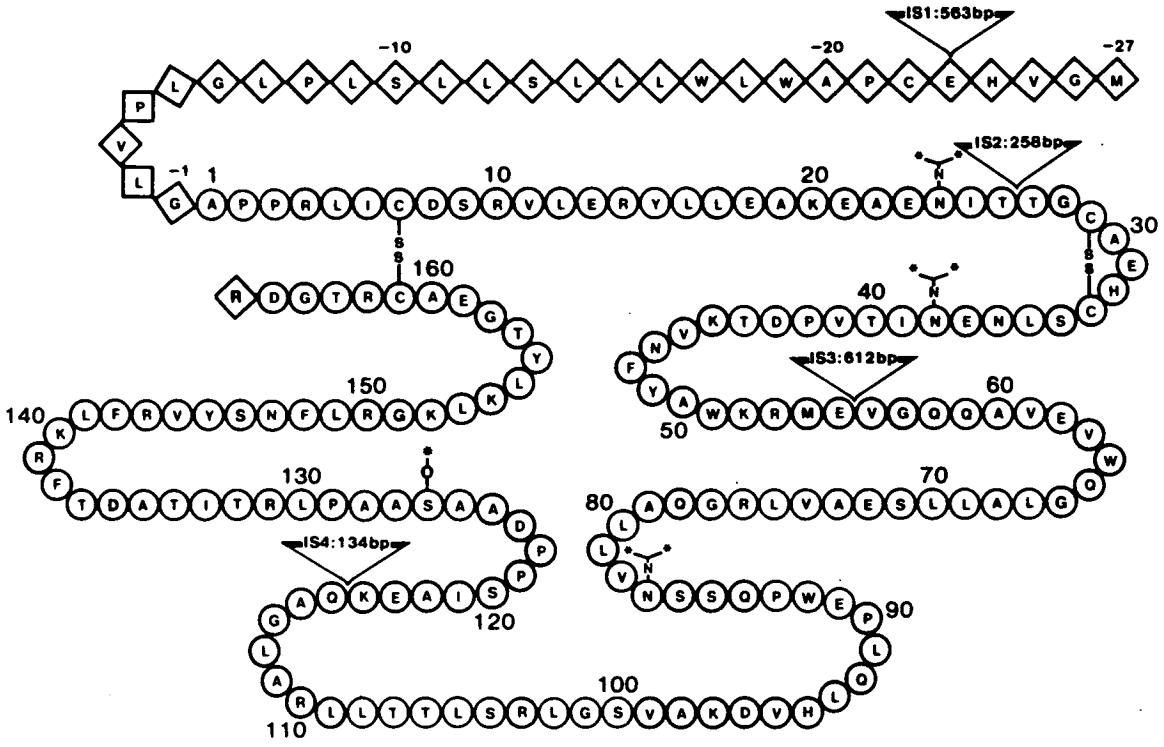



**Figure 8.21** Amino acid sequence of the 193-amino-acid primary translation product of the human erythropoietin (EPO) gene is presented in one-letter code. The 27 amino-terminal amino acids of the putative signal peptide are boxed, and the residues in the 166-amino-acid mature hormone are circled. Positions of the two disulfide bonds (S—S) and the three N-linked glycosidation sites (Y) are noted. Positions at which the four intervening sequences (ISs) interrupt the coding position of the gene are given, along with the length of each IS.The C terminal arginine is removed post-translational yielding a mature product of 165 amino acids. *Reproduced from Browne JT et al. Erythropoietin: Gene cloning, protein structure, and biological properties. Cold Spring Harbor Symp Quant Biol 1986;51:693–702, with permission.*

probes to screen a human cDNA library. The erythropoietin that was finally produced proved to be a posttranslationally modified product of a single-copy gene that was highly conserved in mammalian species. These studies showed that the erythropoietin molecule is a 193-amino-acid primary translation product. The structure, including the 27-amino-acid putative signal peptide, is shown in Figure 8.21. N-linked glycosylation sites appear to be conserved in human, monkey, and mouse erythropoietin and are important to biological activity. When the gene is successfully expressed in somatic cell vectors, carbohydrate modifications apparently occur that enhance biological activity.

### *Clinical Applications*

Patients with end-stage chronic renal failure were logical candidates for the first use of this recombinant hematopoietic hormone. Treatment of 10 patients on thrice-weekly hemodialysis with recombinant erythropoietin was reported by Winearls et al.[182] All patients showed encouraging rises in both reticulocyte numbers and hemoglobin concentration. Use in anephric end-stage patients with renal disease appeared to be particularly appropriate, because during normal homeostasis the hormone is produced by the kidney, and anephric patients have inappropriately low levels of circulating erythropoietin.[183] In such patients a possible complicating

factor might be introduced, since increments in hematocrit might also increase blood viscosity and create additional clotting or coagulation problems in shunts commonly used for arterial access. Initial therapeutic trials appear to be encouraging, but a much longer period of trial and observation is needed before final evaluation of this form of treatment. Additional trials of recombinant erythropoietin given to 25 patients with end-stage renal disease have been reported.[184] Doses ranging from 15 to 500 U/kg of body weight were used, and dose-dependent increments of effective erythropoiesis were recorded. With 500 U/kg, changes in hematocrit of as much as 10% in 3 weeks were noted. Of 18 patients in whom erythropoietin doses proved effective, 12 who had previously required transfusions no longer needed them to maintain hemoglobin and hematocrit values. Four patients showed concomitant increments in serum creatinine and potassium values. No antibodies to the administered erythropoietin were detected. Some improvement in endogenous serum erythropoietin has been noted in a patient switched from hemodialysis to continuous ambulatory peritoneal dialysis.[185] Further long-term comparative assays will be necessary to determine whether the method of dialysis itself affects recombinant erythropoietin metabolism and availability.

The initial trials of recombinant erythropoietin in patients with end-stage renal disease are extremely promising, although other factors, such as shortened red cell survival and blood loss or mechanical trauma during dialysis, may aggravate the anemia in uremic patients. Long-term effects of increased hemoglobin and hematocrit values in patients with chronic renal failure also need to be evaluated. In both initial trials, there were difficulties with blood pressure control in some individual patients. Increasing hematocrit values in dialysis patients may be associated with an increase in peripheral resistance and blood pressure.[186] The possibility of increased problems with arterial and venous access because of increments in blood viscosity must also be considered. Long-term trials with monitoring for parallel changes in blood pressure and hypercoagulability and possible adverse effects of increments in total blood volume should provide a reasonable assessment of the overall benefits of recombinant erythropoietin therapy in patients with end-stage renal disease.

Insight into the effect of recombinant erythropoietin on the anemia of progressive renal failure was provided by a study of 17 patients with anemia and progressive renal failure who did not yet require dialysis.[187] In this trial, the dose of erythropoietin (50 to 150 U/kg body weight) was adjusted to the hematocrit response. All 17 patients showed a positive response to erythropoietin. The rate of beneficial response depended on the initial erythropoietin dose and was similar to what had been observed with patients on dialysis. The rate of decline in renal function did not change as the hematocrit rose, and all patients reported improvements in appetite, activity levels, and sense of well-being. This study showed that the anemia of renal failure was responsive to erythropoietin but that the renal failure itself did not improve as the anemia was corrected.

### *Erythropoietin Levels after Renal Transplantation*

Further analysis of the interaction of decreased renal function, erythropoietin response, and restoration of renal function was reported by Sun and co-workers[188] in a group of 31 renal transplant recipients. These patients were treated with cyclosporine and human serum erythropoietin levels were measured by a radioimmunoassay procedure. The mean serum erythropoietin concentration in the pa-

tients before receiving the transplants was similar to that in normal subjects who were not anemic. After the transplant, a transient ninefold increase in serum erythropoietin was followed by a smaller (threefold) but sustained second elevation. The second increase in erythropoietin level was accompanied by graft recovery and complete resolution of previously existing anemia. Serum erythropoietin levels returned to normal as the patients' hematocrits continued to rise toward normal. Thus, once erythropoiesis is initiated in renal transplant patients, the process is sustained by normal serum erythropoietin levels.

### *Erythropoietin Treatment of Myeloma*

Because anemia is a common complication of multiple myeloma and persists as the disease progresses, erythropoietin was administered to 13 patients with myeloma-associated anemia.[189] The patients received erythropoietin three times a week for 6 months. Eleven of the patients showed steady increases in hemoglobin levels and subsequent correction of the anemia. No adverse side effects were observed and no episodes of hypertension recorded. No changes were recorded during this study in levels of serum M component as a measure of overall tumor burden. Considerable improvement in sense of well-being was recorded by myeloma patients whose hemoglobin levels increased with erythropoietin therapy.

### *Erythropoietin and Megakaryocytes*

The ready availability of recombinant (r) erythropoietin has afforded opportunities to examine its effect on other hematopoietic functions. Two groups have reported an apparent tropic or stimulatory effect of recombinant erythropoietin on human megakaryocyte colony formation[190] or differentiation of murine megakaryocytes in vitro.[191] These effects on other hematopoietic precursors besides erythroid cells raise some interesting questions concerning common signal transductions by many of the hematopoietic stimulatory factors or hormones. As work in this area progresses, it may become apparent that erythropoietin stimulates cells not necessarily restricted to the erythroid lineage.

## Hematopoietic Colony-Stimulating Factors

Regulation of blood cell production and differentiation is one of the most complex examples of control of a system involving mutlilineage development. A common family of pluripotential stem cells located mainly in the bone marrow differentiates into large numbers of erythrocytes, neutrophils, basophils, eosinophils, monocytes, platelets, and lymphocytes, which circulate in the blood or perform selected functions in specific tissues. Many of these blood elements are short-lived and may be called on to expand their numbers rapidly in situations of physiological stress. Work on the specific factors that are necessary for differentiation of normal progenitor cells along multiple parallel but eventually distinct pathways has spanned at least two decades. Development of cell culture systems for the study of clonal growth of selected hematopoietic progenitor cells was essential in understanding the normal differentiation process. This sort of developmental scheme is illustrated in Figure 8.22.[192] Since the various tropic factors were identified through cell culture systems in which uniform colonies were identified, the factors were called hematopoietic colony-stimulating factors (CSFs). Work related to several of these factors including isolation of cDNAs and the genes of four of the major human

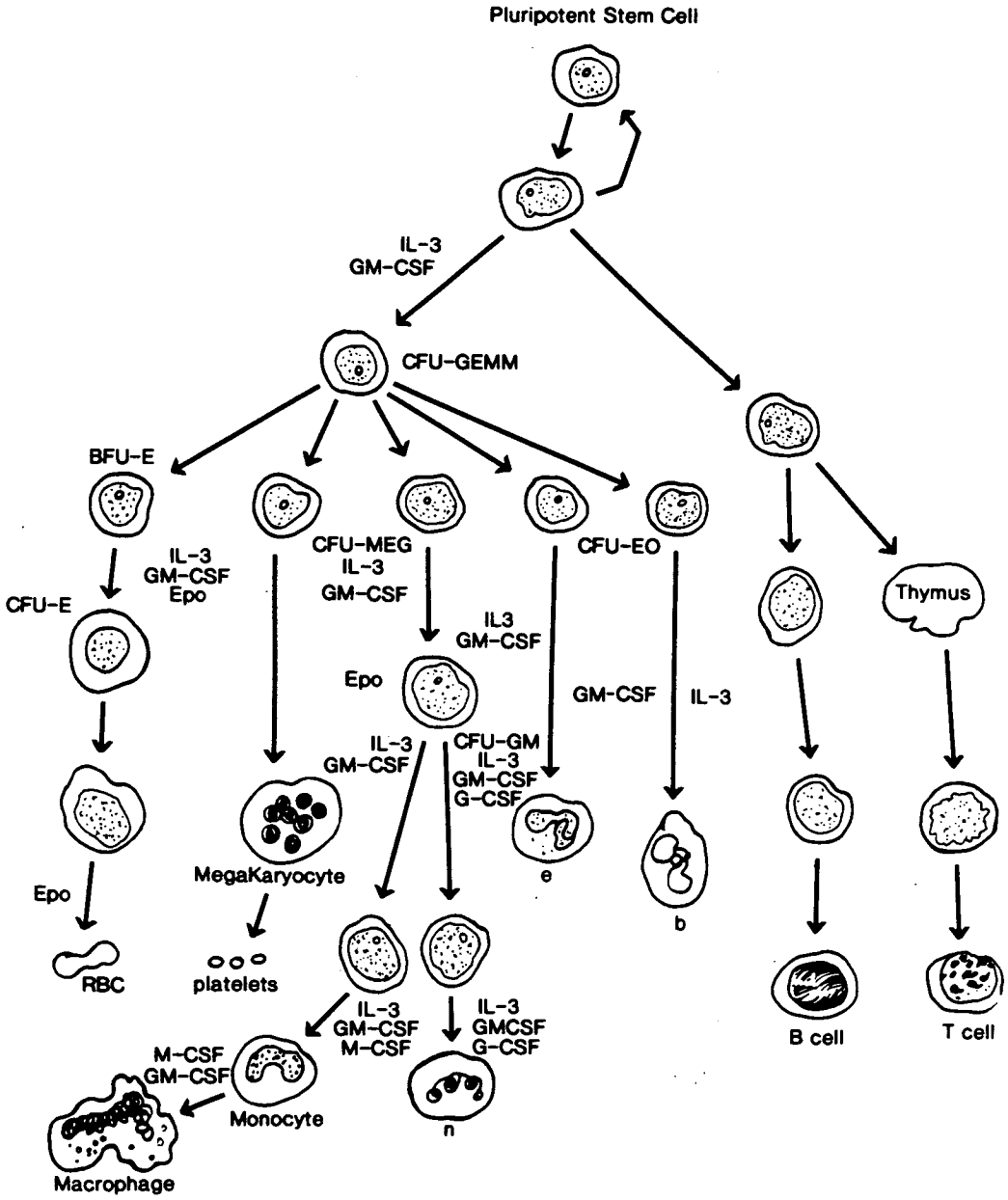



**Figure 8.22** Interactions of the colony-stimulating factors with hematopoietic cells. The progenitor cells that are identified in the culture systems are CFU-GEMM, colony-forming unit granulocyte-erythrocyte-monocyte-megakaryocyte; CFR-Meg, CFU megakaryocyte; CFU-Eo, CFU eosinophil; CFU-GM, CFU granulocyte-monocyte); CFU-E, CFU erythroid, and BFU-E, burst-forming unit erythroid. The abbreviations for the hematopoietic lineages are n, neutrophil; e, eosinophil; b, basophil; m, monocyte/macrophage; E, erythrocyte; and M, megakaryocyte. The interactions of the different CSFs with the various lineages are as indicated. These interactions are based on analysis of mature cells from colonies grown in the presence of the CSFs. The sites of action indicate that at least some but not necessarily all of the progenitors of that lineage are responsive to the indicated CSF. Erythropoietin (Epo) is essential for development of erythroid cells and can promote the differentiation of megakaryocyte progenitors in vitro. *Reproduced from Clark and Kamen,*[192] *with permission.*

myeloid growth factors has rewarded the long and painstaking study of their actions. Moreover, application of several of these growth factors to pressing problems of clinical medicine is already beginning.

### *Colony-Stimulating Factors*

Initially, several different CSFs were distinguished by analyses of individual activities in semisolid growth media. In the mouse system four major types were recorded; two showed relative lineage specificity for granulocytes (G-CSF) or macrophages (M-CSF). By contrast, colonies of cells grown with multi-CSF or interleukin 3 (IL-3) were sometimes found to contain many different cell lineages.[193] Stimulation of stem cells with both GM-CSFs (granulocyte-macrophage colony-stimulating factors) induced neutrophils, macrophages, eosinophils, and other cell types.[194] The theoretical model for the operation of the various growth factors presumes that G-CSF and M-CSF support growth and division of relatively late progenitors already committed to their own lineage differentiation pathways. The GM-CSF is thought to act on earlier progenitor cells that are still capable of differentiating into neutrophils, eosinophils, or monocytes. Within the human system, a family of four similar factors has been recognized as G-, B-, GM-, and multi-CSF.[194,195] A number of other growth factors that affect important differentiation pathways have also been recognized. Thus, as noted earlier, erythropoietin functions in early erythroid cell development and may have some effect on megakaryocytes. Interleukin 2 (IL-2) is an important growth factor for both T and B lymphocytes.[196] Another tropic factor, hematopoietin 1,[197] is thought to act on stem cells to allow them to become responsive to other CSFs; a similar function has also been attributed to interleukin I (IL-1).

### *Molecular Cloning of Human Colony-Stimulating Factor*

Successful cloning of the four major CSFs has been a major practical achievement in the application of molecular biological techniques. Each separate factor was identified and cloned through a large collaborative effort involving cell biologists, protein chemists, and molecular biologists. Each CSF was known to exist in only trace amounts, making its biochemical characterization very difficult. The trace levels of the CSF molecules were naturally reflected in correspondingly low levels of mRNA in the tissues or cell lines examined, which made their cloning exceedingly difficult.

Complementary DNAs for G-CSF[198,199] and M-CSF[200] were obtained by a general strategy in which purification to homogeneity was followed by amino acid sequencing. The derived sequences were utilized to predict nucleotide sequence in several different mRNA regions, and synthetic oligonucleotides were prepared to identify cDNAs encoding the entire CSF molecules. The sequences derived were then checked by expression in mammalian cells to recover the respective biological activities. In the case of human M-CSF, much manipulation of the initial cDNA clones was necessary before a final functional product was obtained.

The cDNAs for human GM-CSF[201] and murine IL-3[202] were obtained by a different approach. Because of their original presence in trace or only picomolar amounts, purification to homogeneity proved very difficult. On the other hand, both of these factors showed uniquely potent biological activities. The high specific biological activity could be exploited by identifying cDNA clones that would direct expression of the desired CSF product after introduction into mammalian cells with

appropriate expression vectors. cDNAs for both human GM-CSF and murine IL-3 were obtained in this manner. This was done by testing pools of plasmid cDNAs for their ability to express the biological activities when introduced into target COS-1 cells.

The human and murine IL-3 genes had very similar structures,[202] containing five relatively small coding exons separated by one large and three small introns. Nucleotide sequence homology was distributed relatively evenly over the 3000 base pairs making up the genes. The human GM-CSF and IL-3 genes have been mapped to the same band on the long arm of chromosome 5.[203] The same area has been found to contain the M-CSF gene and its receptor, the proto-oncogene, c-*fms*, as well as the genes for several other growth factors and receptors. The significance of this clustering of genes for growth factors on chromosome 5 is not clear, but it must be important in terms of basic mechanisms of cellular differentiation.

### *Regulation of Colony-Stimulating Factor Gene Expression*

Much remains to be learned about the ways in which the CSF genes are regulated or modulated during normal events in vivo. It is well known that absolute numbers of various blood cells, such as polymorphonuclear leukocytes or monocytes, may change rapidly in response to extrinsic signals such as stress or infection. Many normal cells do not appear to have detectable levels of mRNA for G-CSF, GM-CSF, or IL-3, but several different cell types have been shown to express CSF genes after activation. Thus, monocytes activated by endotoxin or γ-interferon express high levels of mRNA for G-CSF and M-CSF but not for GM-CSF.[204] In parallel, IL-1 and tumor necrosis factor, which are potent lymphokine products of monocytes or macrophages, can activate G-CSF and GM-CSF in various body cells, including fibroblasts and endothelial cells.[205,206] Thus, signals released by stimuli such as infection, trauma, or other changes in the internal body milieu must be capable of up-regulating gene expression and mRNA coding for these potent CSF materials. The cellular source of these factors that maintain normal steady-state hematopoiesis has not been completely identified. By contrast, the mRNA for M-CSF is readily observed in a variety of normal cells and tissues. Such extensive representation in many tissues may indicate its importance in survival and the need to provide mechanisms for instant production of monocytes and macrophages at many tissue sites.

### *Recombinant Colony-Stimulating Factors—Structure and Biological Action*

A number of studies have indicated that recombinant CSF products contain essential structural elements that are very similar to natural molecules produced in vivo. In several cases, such as GM-CSF and IL-3, the molecular size of the products studied indicates that up to 50% of the mass of the protein may be carbohydrate.[198–201] The functions of the extensive carbohydrate modifications of GM-CSF and IL-3 are still not understood. The addition of carbohydrate residues may influence body compartment distribution or catabolism. More information related to the latter is needed.

Availability of four human CSF proteins has made it possible to examine their biological activities in some detail. Recombinant G-CSF is a lineage-specific factor that supports proliferation of neutrophils. It also has a marked stimulatory effect on biological activities of neutrophils by increasing superoxide anion production, ability to kill tumor cells in antibody-dependent cytotoxicity, or ability to phag-

ocytize particles.[207,208] Recombinant GM-CSF supports proliferation of macrophages and eosinophil colonies in vitro as well as colonies containing both neutrophils and macrophages.[207] It has also been shown to serve as a growth factor for the leukemia cells of many patients with chronic and acute myeloid leukemia.[208,209] There is no direct evidence that exaggerated expression of the GM-CSF gene is directly related to acute myelocytic leukemia, but patients have been reported whose early leukemic cells are autocrine for GM-CSF, indicating a built-in stimulus for continued growth.[208]

Studies with recombinant gibbon IL-3 indicate proliferation of myeloid progenitor cells as well as those of erythrocytes, neutrophils, eosinophils, basophils, macrophages, and megakaryocytes. It appears that this factor acts on earlier progenitor cells than GM-CSF or G-CSF. The activity of recombinant human M-CSF is still under study. Although the material strongly stimulates murine macrophage colony formation, it has been less active with human target cell systems. The recombinant form is a powerful activator of human macrophage cytotoxicity.

### *In Vivo Therapeutic and Clinical Trials*

The potential usefulness of CSFs in a number of clinical situations has excited a great deal of hope and positive expectations. Such recombinant proteins might be useful in restoration of hematopoietic dysfunction after immunosuppression or irradiation and in augmenting host response to overwhelming infection.[210] The same factors might be very effective in increasing levels of primed effector cells that could help rid the host of malignant cells.[211] Suppression of myelopoiesis after chemotherapy is a common feature of many otherwise effective antitumor programs and is one of the major adverse effects of such treatment. Limited phase I trials of G-CSF have begun in such patients, and the results appear extremely promising.

Matoyoshi et al.[211] in Japan reported positive effects of partially purified human urinary colony-stimulating factor preparations on granulocytopenia after antitumor chemotherapy. This initial trial produced distinct increments in peripheral blood granulocytes in treated subjects but did not affect levels of erythrocytes, platelets, monocytes, or lymphocytes. Human granulocyte CSF was administered to normal and cyclophosphamide-treated primates.[212] The G-CSF was administered by two daily subcutaneous injections for a period of 2 to 4 weeks. A dramatic increase in neutrophils was noted 24 hours after initiation of treatment; increases in white blood cell counts were due primarily to increments in the absolute numbers of neutrophils. These changes are illustrated in Figure 8.23. Similar dramatic increases in circulating neutrophils were demonstrated in animals pretreated with cyclophosphamide. In these animals, treatment with G-CSF shortened the time period of white blood cell recovery to 1 week, compared to 4 weeks in control myelosuppressed monkeys. Recombinant GM-CSF was also given to 16 patients with acquired immunodeficiency syndrome (AIDS) and leukopenia by Groopman and co-workers.[213] Treatment consisted of an initial intravenous dose of $1.3 \times 10^3$ to $2.0 \times 10^4$ U/kg. Forty-eight hours after the initial dose, a continuous intravenous infusion was given. The GM-CSF produced a dose-dependent increase in counts of circulating white blood cells and of neutrophils, eosinophils, and monocytes, with peak leukocyte counts ranging from $4575 \pm 2397$ to 48,700 in patients on the lowest to highest doses. Mild side effects of flushing, myalgia, and phlebitis were noted in some patients but no severe adverse reactions were recorded. These

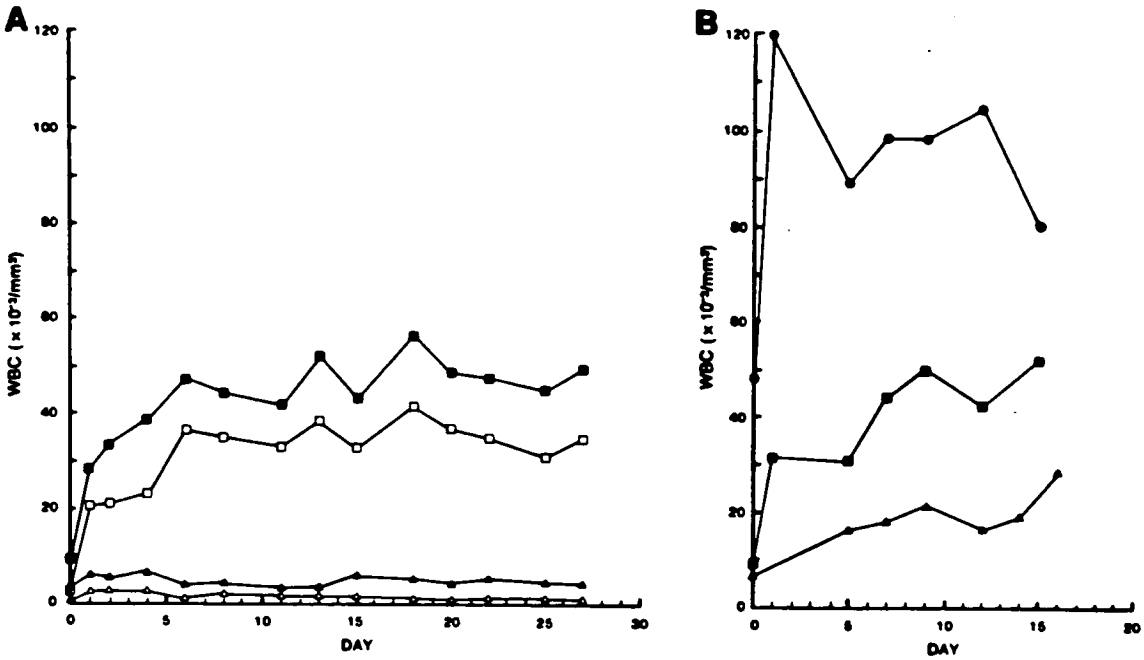



Figure 8.23 Time course of peripheral white blood cell (WBC) count and absolute neutrophil count (ANC) of healthy cynomolgus monkeys treated with recombinant human G-CSF and a control monkey receivng only buffer. (A) WBC level (■) and ANC (□) of a healthy monkey treated with rhG-CSF at 10 μg/kg/d subcutaneously in two daily doses. The WBC level (▲) and ANC (△) of the control monkey are also shown. (B) Effects of different concentrations of rhG-CSF: (▲) 1 μg/kg/d, (■) 10 μ/kg/d, and (●) 100 μg/kg/d. The monkey receiving 10 μg/kg/d was subsequently treated with 100 μg/kg/d, and therefore day 0 of 100 μg/kg/d corresponds to day 10 of 10 μg/kg/d. *Reproduced from Welte et al,*[212] *with permission.*

initial results were encouraging and suggested that GM-CSF was relatively well tolerated and could induce rapid increments in leukocyte counts in such immunosuppressed patients. Furthermore, it indicated that patients with serious infections could respond to the agent even though relative myelosuppression was a feature of the underlying AIDS disorder.

A second therapeutic trial of recombinant human GM-CSF has been reported in patients with myelodysplastic syndromes.[214] As part of a phase I trial, GM-CSF was administered to eight patients by continuous intravenous infusion for 2 weeks and again after a 2-week rest. Treatment was associated with 5- to 70-fold increases in peripheral blood leukocytes, including elevations of granulocytes in all eight patients. Absolute numbers of monocytes, eosinophils, and lymphocytes also increased in all patients. Three of the patients showed 2- to 10-fold increments in platelet counts and improved erythropoiesis to the extent that two patients who had required red cell and platelet transfusions were able to discontinue such replacement. Examination of bone marrow populations showed a relative decrease in percentages of blasts and an increase in ratio of differentiated myeloid cells to immune cells of the same lineage. Bone pain was experienced by some patients and was dose limiting when associated with high white blood cell counts. Of great interest in this study was the apparent multilineage stimulation in the patients treated. Although experimental in vitro and in vivo work had indicated that GM-CSF was potentially a multilineage hematopoietic stimulator, it is possible that

neutrophil counts from 717 ± 171 to 9814 ± 2198 per microliter ($P = 0.009$). Although in five of the six patients treated, cycling of blood cell counts continued, the length of the relative leukopenic period decreased from 21 to 14 days. The neutrophil turnover increased almost fourfold as measured by kinetics of labeled autologous blood neutrophils. Overall, the G-CSF therapy appeared to be effective in decreasing the frequency of oropharyngeal inflammation, fever, and infections in the cyclic neutropenia group. A few patients experienced mild bone pain after intravenous administration of G-CSF, and a consistent mild elevation of leukocyte alkaline phosphatase to levels above 200 was observed in all patients treated (normal 60 to 140 units). A moderate increase in splenic size was noted in all six patients.

Treatment success in a marrow stem cell disorder such as cyclic neutropenia is a substantial positive result in terms of therapeutic application. However, there are some lingering questions that a longer period of sequential observations may answer. The initial euphoria that prevailed shortly after the clinical introduction of GM-CSF and G-CSF has waned somewhat, but their widespread use clearly has led to beneficial results in a substantial proportion of patients. The early studies with GM-CSF indicated that this recombinant product could accelerate the leukemic process.[221] Also, in vitro studies have indicated that G-CSF may stimulate cell lines for colon cancer and small cell lung carcinoma.[222] Continued experience will help to assign their overall value in patients with various stem cell disorders.[222,223]

As more experience is gained with these agents a more seasoned evaluation of their usefulness and possible untoward effects will eventually become possible.

## Tissue Plasminogen Activator

During the last several years there has been interest in dissolving clots in the therapy of acute vascular disorders such as myocardial infarction or deep vein thrombophlebitis. Therapeutic trials of streptococcus-derived products such as streptokinase are ongoing.[224] Plasminogen activators, streptokinase, and urokinase, which were the first agents available for clinical use, were effective in dissolving thrombi and emboli but were not specific for fibrin. These agents could activate plasminogen in the circulation as well as the plasminogen bound to fibrin in the clot. Activation of plasminogen in the circulation increased the proteolytic activity of the blood and produced degradation of fibrinogen and clotting factors V and VII. This led to an increased risk of bleeding complications in patients treated with these agents.

For these reasons, efforts were directed at manufacturing the naturally occurring body protein that activated blood clot dissolution, tissue plasminogen activator (t-PA). When small amounts became available, a number of trials were undertaken.[225] Experience thus far shows that this agent binds strongly to fibrin but avoids some of the hemostatic defects. Moreover, it has a very short half-life in the circulation. Despite the milder hemostatic defect observed in t-PA compared to streptokinase, the incidences of bleeding complications in parallel trials have been approximately equal.[226] Because t-PA represents a native recombinant protein, the potential problem with antigenicity and subsequent reactions appears to be relatively excluded. The rapid development and widespread use of this important recombinant product are yet another example of the remarkable spread of recombinant DNA methodology and its application to significant common medical problems. Whether recombinant t-PA will prove to be a more effective therapeutic

such an effect was also mediated by other lymphokines such as IL-1 released in vivo as a result of the GM-CSF. As the authors point out, because acute leukemia develops in 15% to 40% of patients with myelodysplastic syndrome[215] and GM-CSF is capable of stimulating leukemic cells in vitro, there was some concern that GM-CSF administration might augment or exacerbate any underlying leukemic aspect of the disease. On the contrary, no changes in proportions of immature blast forms and an actual improvement in the spectrum of differentiation were recorded during this initial study.

The method of continuous intravenous administration was chosen in this study because previous studies in nonhuman primates had indicated a relatively short half-life of the active material. The initial results suggest that these recombinant products may have great therapeutic potential as their use is cautiously expanded. CSFs could potentially be utilized to supply large numbers of primed effector cells to sites of infection or tumor tissues.

Recombinant GM-CSF used after high-dose chemotherapy and autologous bone marrow transplantation[216] in 19 patients with breast cancer or melanoma accelerated total leukocyte and granulocyte recovery. Toxic effects in this trial group appeared to be relatively mild and included some edema, weight gain, or myalgias. Generalized edema, pleural effusions, and hypotension were observed in two patients. The results again appeared to be encouraging and showed that recombinant GM-CSF was capable of accelerating myeloid recovery in such patients. A similar study was reported by Sheridan and co-workers,[217] who administered G-CSF by continuous subcutaneous infusion to 15 patients with nonmyeloid malignancies treated with high-dose chemotherapy and autologous bone marrow infusion. Sustained levels of serum G-CSF were found. Recovery of neutrophils was accelerated in the G-CSF–treated patients compared with historical controls. This resulted in significantly fewer days of parenteral antibiotic therapy.

Recombinant G-CSF was used in parallel with chemotherapy for 27 patients with transitional T-cell carcinoma of the urothelium.[218] The protocol resulted in a dose-dependent increase in absolute neutrophil counts and reduced the number of days during which patients' absolute neutrophil counts remained below 1000 per microliter. It appeared to be capable of reducing both the hematopoietic and the oral toxicity of chemotherapy.

Another aspect of possible therapeutic benefit has been explored in experiments by Silberstein et al.[219] They found that recombinant GM-CSF enhanced eosinophil cytotoxicity against schistosomula sixfold. Moreover, GM-CSF increased the generation of leukotriene C4 by a mean of 135%. These findings indicated that GM-CSF could act to mediate T lymphocyte regulation of eosinophil functions. Because eosinophil-mediated cytotoxicity has been considered a possible protective mechanism whereby the host reacts against parasitic forms such as schistosomes, it is conceivable that administration of GM-CSF might find some use in the therapy of such parasitic diseases.

### *Cyclic Neutropenia*

Six patients with cyclic neutropenia were treated with recombinant human granulocyte CSF for periods of 3 to 15 months.[220] All the patients showed aphthous stomatitis, pharyngitis, lymphadenopathy, febrile episodes, and frequent infections associated with periods of neutropenia. Daily intravenous or subcutaneous administration of G-CSF at 3 to 10 μg/kg body weight resulted in an increase in mean

approach to clot dissolution in acute myocardial infarction or pulmonary embolism than streptokinase will be known after a much longer period of evaluation and monitoring. The important aspect of t-PA in the context of the subjects addressed in this volume is that it is an outstanding example of the power of recombinant DNA technology to focus on an important clinical need.

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